A STUDY OF THE RELATIONSHIPS BETWEEN CHROMATOGRAPHIC BEHAVIOR AND STRUCTURE OF STEROID HORMONES

By

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This dissertation is dedicated to my husband.

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INTRODUCTION

Statement of Purpose

This study had two main purposes:

The investigation of the gas-liquid chromatographic behavior of a series of isomeric androstanes with the aim of correlating chemical structure to chromatographic mobility.

Through the knowledge and insight gained in the course of the above study to apply the gas-liquid chromatographic method as an analytical technique in the isolation and measurement of C_{19} steroids of importance in man. The ultimate objective was the actual measurement of these steroids in biological fluids.

In the first part of the study twenty-eight isomeric androstanes were utilized. These were chosen on the basis of their particular structural relationships. The structures ranged from the unsubstituted androstane nucleus to the disubstituted steroid. The substituent was an oxygen function in all cases.

For the separation study of biologically significant androstanes, two pregnane isomers were included which occur together with the C_{19} steroids in biological fluids.

Chromatography

Historical Background

Tswett is generally given credit for the invention of the method of chromatography, although in 1850 a German dye chemist, F. F. Runge, had described a separation process which might be classified as paper chromatography (1). In 1903 Tswett (2) separated the pigments of green leaves into several components on an adsorbent column of calcium carbonate. Since this separation by selective adsorption involved the formation of colored bands on the column, Tswett named the process chromatography. The process has since been extensively applied to colorless materials, thus the term chromatography is a misnomer.

The purpose of chromatography is the separation of closely similar substances. Most separations depend upon the distribution of substances between two phases. One phase is fixed and may be liquid or solid and the other phase is mobile and may be liquid or gas. The phases must be selected so as to enhance the maximum difference between the distribution of the desired substance and that of the other materials. The particularly distinguishing feature of chromatography is the amplification of the distribution process. This amplification is accomplished by the intimate contact of each element of the moving phase with each element of the very finely divided fixed phase. The

process of continuous and successive contacts results in the distribution of the solute between the elements of the two phases and the attainment of equilibrium in a very small length of time.

The two basic types of chromatography are adsorption and partition and are distinguished by the nature of the process of distribution of the solute between the phases. (See Figure 1.) In adsorption systems, the distribution is dependent on the concentration of the substance that is being distributed. At any one temperature, the adsorption isotherm, represented as the function of the quantity of solute in one phase and the total amount in the system is a curve. The adsorption isotherm is effected by the presence of other substances in the system. This is probably due to the limited surface area of the solid phase. In contrast to the adsorption isotherms, the isotherms for the partition systems (liquid-gas or liquid-liquid) are linear for a wide range of concentration of the solute as well as for moderate concentrations of other substances in the system. This is the major distinguishing feature of partition system and accounts for their tremendous success (3).

With the exception of the work of Runge, early phases of the development of chromatography were limited to column chromatography. In the steroid field both adsorption and partition methods found wide application.

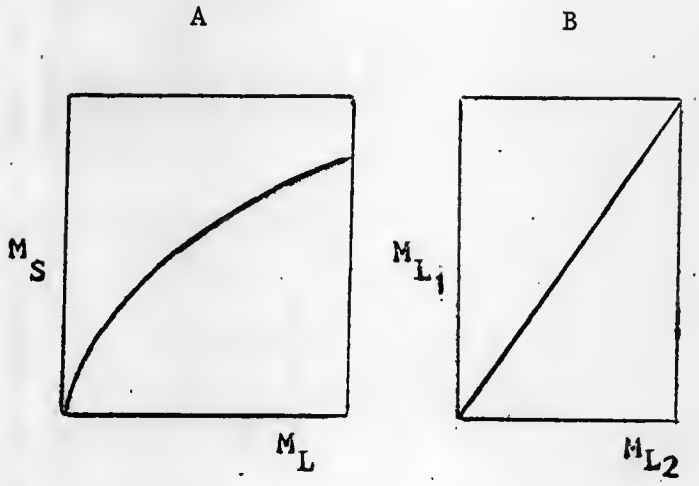

FIGURE 1

Distribution isotherms:

A Adsorption isotherm.

B Partition isotherm.

Two types of adsorption methods were employed by the early investigators. The first type involved the use of large columns for large samples and were primarily preparative in purpose (4,5). The second type was for separation and estimation of small quantities of samples on small columns. This method found application in the separation and estimation of urinary 17-ketosteroids (6,7) and of adrenal steroids and metabolites (8). Alumina, magnesia and silica gel were used most frequently for column packing.

The first successful partition methods were developed by Butt, Morris and Morris (9,10) who utilized Celite columns. Shortly after the publication of these methods, intensive research was directed towards partition chromatography techniques. The problem of the low solubility of steroids in systems based on water as the stationary phase was overcome by the development of organic systems and a whole new field of partition chromatography of steroids became available. Among the pioneers in this field were Morris and his group (9,10), Zaffaroni and his associates (11), Heftmann(12), Bush (13,14) and Savard (15) to name a few.

The systems of partition chromatography consisted of three components: a major component of the mobile phase which was relatively non-polar, a component of the stationary phase more or less polar in character to enhance the solubility of the steroid and a third component of a small amount of water. The water was later omitted from systems using impregnated paper and from certain column methods. In

reversed-phase partition chromatography systems, the mobile phase was more polar than the stationary phase and the support was hydrophobic.

The principles of liquid-liquid and gas-liquid partition chromatography were described by Martin and Synge in 1941 (16) but the concept of a gaseous moving phase was not applied until 1952 when James and Martin published their now classical paper (17). Since that time, extensive research on gas chromatography has been carried out in laboratories throughout the world for many types of problems. The great interest in various forms of gas chromatography is due to its wide range of application, speed of analysis and the sensitivity of detection for micro-quantities of sample.

Gas Chromatographic Methods

Gas chromatography is the process by which a mixture is separated into its individual components by a gaseous mobile phase. If the stationary phase is liquid, the method is called gas-liquid chromatography and the separation is due to the differential solubility of the materials between the liquid and gas. If the stationary phase is a solid, the method is called gas-solid chromatography and the separation is due to the adsorption of the sample components on the solid surface.

The two types of gas chromatographic methods are further distinguished by the manner in which the sample moves through the column.

Elution, displacement and frontal analyses are three techniques which may be used in gas-solid chromatography. The elution technique is used almost exclusively in gas-liquid chromatography.

Frontal analysis is the passage of a continuous flow of carrier gas containing the sample mixture through a column of adsorbent. The idea of using a continuous stream of the sample itself as a displacer where each solute attains its own competitive adsorption equilibrium was the contribution of Tiselius (18). Separation is achieved only for that component which emerges first from the column, consequently frontal analysis is of limited use as a method of separation.

Displacement analysis, which was elaborated by Claesson (19), is the method of displacing the sample already on the column by means of passing a continuous flow of a carrier gas which contains the vapor of a substance called the displacer. The displacer is so chosen that it is more strongly adsorbed onto the column than any component of the sample mixture. The component displaced by the displacer in turn displaces another component of the sample that is less strongly adsorbed. Thus the components of a sample move along the column at the same rate that the displacer is saturating the column behind the sample.

Elution chromatography involves the continuous passage of an unadsorbed gas or liquid through the chromatographic system. A volatilized sample is introduced into this stream of gas and is carried

down the column. Components of the sample distribute themselves between the mobile gaseous phase and the stationary phase. Each component eventually emerges from the column after the passage of a characteristic volume of carrier gas. The unique feature of elution technique, as compared to frontal and displacement analysis, is the emergence of the solute as a peak. The retention time t_R, defined as the length of time between the introduction of the sample into the chromatographic system and the emergence of the peak maximum, is a means of qualitative identification of the compound. The peak area can usually be related directly to the concentration of the compound.

Principles of Gas Chromatography

A distinguishing feature of gas-chromatography is the utilization of high temperatures in order to maintain the solutes in the vapor state. The wide selection of liquid phases enhances the applicability of gas-liquid chromatography methods to a wide range of analyses. The technique of gas-liquid chromatography is similar to liquid-liquid partition and the gas-liquid column is analogous to a distillation column. However, there is one major difference between the two systems, which is of great practical importance. In systems of gas-liquid chromatography there is no interference with separation due to azeotropy. At the inlet of a gas-liquid chromatography column there might occur an interaction between the components of the sample but over the whole length of the column, the interaction of these components with the

liquid phase far outweigh any mutual effects (20). Thus each component is carried through the column independently of any other component.

A theoretical plate treatment is applicable to both liquid-liquid and gas-liquid columns. A theoretical plate is defined as a section of the column in which the vapor leaving the section has the composition that would be in equilibrium with the average concentration of liquid solution within that section. The most important assumption underlying the theoretical description of the chromatographic process is that equilibrium between the two phases is obtained after each minute movement of the mobile phase.

The separation per plate (separation factor) and the total number of plates (N) existing in a column determine the over-all separation achieved. The temperature of the column and the nature of the stationary phase of the components to be analyzed all determine the separation factor. This factor is defined as the ratio of the retention times of two components to each other. Separations can be improved by the use of a liquid phase with specific characteristics, such as selective retention of compounds containing double bonds or ketone groups.

The length of column in which equilibrium occurs is defined as the height of an effective theoretical plate (16). The number of theoretical plates exhibited on a column for a specific liquid phase,

solute and temperature can be calculated in a number of ways all of which are a measure of the degree of peak spread relative to its residence in the column. The length and diameter of the column, the diffusion constant of the solute in the two phases, the amount of stationary phase, uniformity of column packing, the rate of flow, temperature and the nature of the carrier gas all are variables affecting the value of N. In general, N is increased by an increase in length of column and a decrease in its diameter.

The equation commonly used for the calculations of N is

$$N = \frac{16\ (t_R)^2}{\Delta t}$$

where t_R is the retention time measured from the introduction of the sample and Δt is the width of peak base. (See Figure 2.) The units may be time or length provided both t_R and Δt are in the same units. This equation for N assumes that the component being studied is in equilibrium with the column. N calculated in this manner is significant only for symmetrical peaks since the above equation is based on probability theory(21).

The height equivalent to a theoretical plate is given by the equation

$$\bar{H} = L / N$$

where L is the length of the column, usually in millimeters. The value of $\bar{H}$ is generally low for columns operating at high efficiency and decreases with increases in retention time. The plate theory provides a useful means of evaluating column efficiency. The

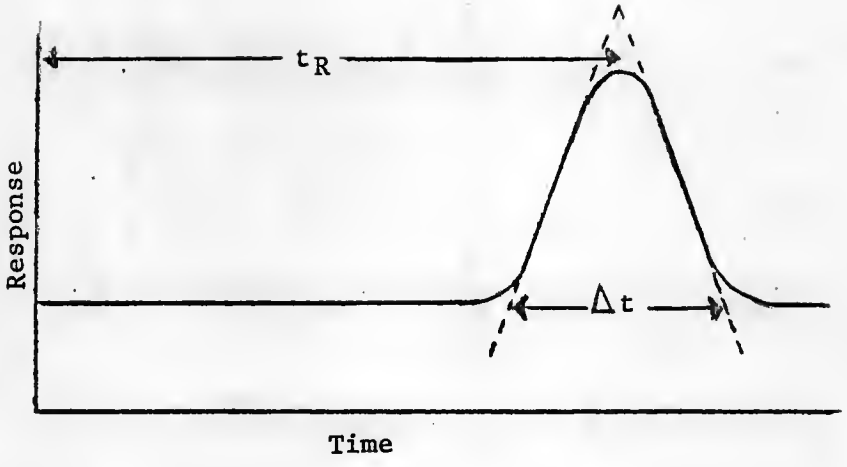

FIGURE 2

A method for calculating number of theoretical plates.

relationship of various terms which determine the value of $\bar{H}$ are given in the van Deemter equation (22).

$$\bar{H} = 2\lambda d_p + \frac{2\gamma D_{gas}}{u} + \frac{8k'}{\pi^2(1+k')^2} \frac{d_f^2}{D_{liq}}$$

where λ is a quantity characteristic of the packing, d_p is the average particle diameter, γ is a correction factor for the tortuosity of the path of the gas in the interparticle space, D_{gas} is the diffusion coefficient of the solute in the gaseous phase, u is the linear gas velocity, k' is the ratio of the fraction of sample in the liquid phase to the fraction in the vapor phase, d_f is the thickness of the liquid film, and D_{liq} is the diffusion coefficient of the solute in the liquid phase.

In the simplified van Deemter equation

$$\bar{H} = A + B/u + Cu$$

A represents the eddy diffusion term, also called the multiple path effect, B/u term is the molecular diffusion and Cu is the term that describes the resistance to mass transfer. The eddy diffusion is the result of the irregular path which the gas follows through the packed column. The irregularities in this path contribute to a broadening of the chromatographic band. A reduction in particle size (d_p), uniform packing and uniform particle size tend to decrease the contribution to $\bar{H}$ of this term. The second factor is the diffusion of the sample molecules in the carrier gas, which results in band broadening. The lower the molecular weight of the carrier gas, the greater is this

factor. Diffusion increases with increase in temperature. An increase in pressure, on the other hand, by increasing the density of the gas, tends to decrease the diffusion. For this reason, high temperatures are employed together with high pressures of the carrier gas.

The term Cu, resistance to mass transfer, includes several parameters. The value of k' is dependent on the partition coefficient as well as the relative volumes of the liquid and the gaseous phase in the column. k' is generally greater than one because the solubility of the solute in the liquid phase is usually greater than in the gaseous phase. Thus any increase in the solubility of the sample in the liquid phase will decrease $\bar{H}$ by a small amount. The d_f term, defined as the effective thickness of the liquid film, is raised to the second power in the van Deemter equation. Its contribution will therefore be appreciable. A reduction in d_f results in increased column efficiency, which may be counteracted by a simultaneous decrease in k' if the amount of liquid phase is reduced. The liquid diffusion coefficient (D_{liq}) is dependent on the viscosity of the liquid phase. An increase in temperature might be expected to reduce the viscosity but since increased temperatures affect k' and D_{gas} adversely, the net result is difficult to assess.

The last term in the van Deemter equation is the rate of flow of the carrier gas. Theoretically eddy diffusion is independent of the rate of flow. The contribution to $\bar{H}$ of molecular diffusion is in inverse ratio to the rate of flow. Resistance to mass transfer,

however, varies directly with the velocity of the gas. If all other variables are kept constant, there will exist an optimum rate of flow for the most efficient operation of a column as indicated in Figure 3. At flow rates less than the optimum value, the contribution of molecular diffusion to $\bar{H}$ will be appreciable. Above optimum flow rate resistance to mass transfer becomes important. However, this part of the curve is relatively flat and operations above optimum velocity will not have too deleterious an effect on $\bar{H}$. The molecular diffusion term is large for carrier gases of high diffusibility. Resistance to mass transfer will be dominant for liquids of high viscosity and for high concentrations of stationary phase. Qualitative and quantitative predictions of the effect of $\bar{H}$ of changes in the parameters of the van Deemter equation are summarized by Patton (23), Bohemen and Purnell (24) and Purnell (25).

For any packed column there exists a pressure drop between the inlet and the outlet ends of the column. An increase in the absolute pressure at either end of the column or the use of a tapered column with a widening diameter towards the outlet end are possible solutions to minimizing the pressure drop. A correction factor for the pressure drop across the column has been derived by James and Martin (17,26). The retention volume defined as

$$V_R = F_c t_R$$

where F_c is the volumetric flow rate of gas, can be corrected to give the limiting retention volume which is independent of pressure (27).

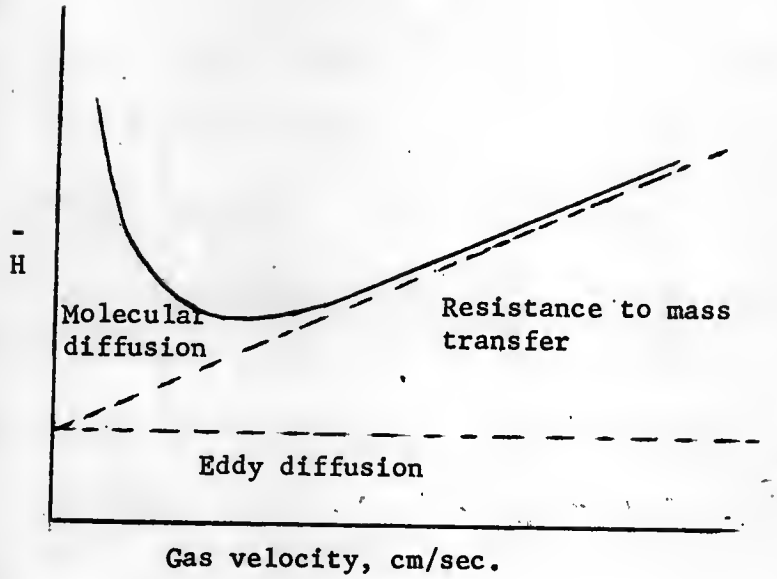

FIGURE 3

Influence of carrier gas velocity on column efficiency.

$$V_R^o / V_R = p_o / \bar{p}$$

where V_R^o is the limiting retention volume at zero pressure drop across the column, V_R is the retention volume, p_o is the column pressure at the outlet end and $\bar{p}$ is the average column pressure.

Various experiments have been devised to test the applicability of the van Deemter theory and will be considered in the Discussion section. The theory has been useful in attaining high column efficiencies.

Basic Gas Chromatographic Apparatus

The apparatus for any gas chromatographic separation consists of a carrier gas supply, a flow regulator, a pressure gauge, a recorder, sample port and a pipet for introducing the sample, a column, a detector and heating units for the last three items.

Carrier gas is supplied from a compressed source and its flow rate through the column is regulated by a pressure valve. The gas passes through the detector and proceeds to the sample port. The sample is introduced into the sample port where it is heated to effect volatilization. The carrier gas carries the sample vapor into the column where the distribution process takes place. The separated components of the sample in the carrier gas enter the detector and pass out to atmosphere or to a collection apparatus for further study. Temperature control of the apparatus components is an important consideration in gas chromatography. The minimum detector temperature

should be that of the column to prevent condensation of solutes which would result in change in concentration. The sample port temperatures should be adjustable to permit the development of temperatures sufficiently high for the rapid vaporization of solutes.

Androstanes

Androstanes belong to the broad group of biologically important organic compounds called the steroids which possess the cyclopentanoperhydrophenanthrene nucleus containing 19 carbon atoms. (This nomenclature includes members previously designated as etiocholanes.) The nucleus contains 17 carbon atoms which make up the condensed system of four rings (Figure 4). The rings are designated as A, B, C and D and the carbon atoms are numbered as indicated in the above structure. The two angular methyl groups (designated as carbon atoms 18 and 19) project to the front of the steroid molecule. This projection is described as β-oriented and is represented by a solid line. Similarly, any projection to the rear of the ring system is designated as α-oriented and is represented by a dotted line.

Conformations of Androstane Ring System

The term conformation describes the different spatial arrangements of the atoms in a single classical structure or configuration. Transformations from one arrangement to another take place by the simple rotation of a single bond. The best known examples are the chair and boat forms of the cyclohexane model (Figure 5). The differences in energy which are primarily due to differences in the

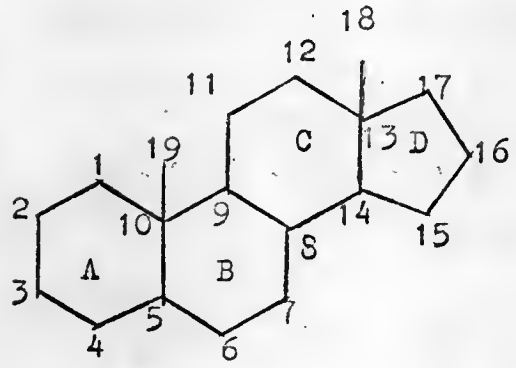

FIGURE 4

Steroid nucleus.

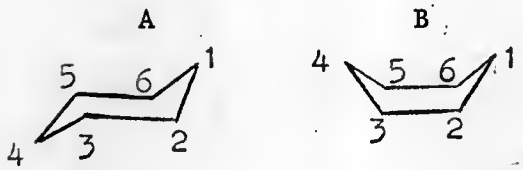

FIGURE 5

Conformations of cyclohexane: A chair, B boat.

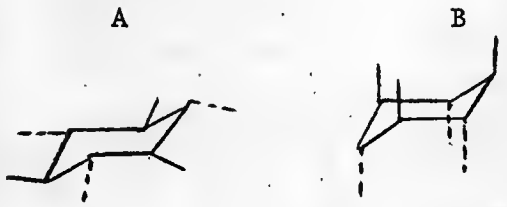

FIGURE 6

A - Equatorial bonds. B - Axial bonds.

repulsions between closely approaching atoms among various conformations determine the stability of the particular conformation. In general, it may be stated that even though reactions often proceed through unstable forms, the unexcited molecules exist in the conformation of lowest energy and therefore of highest stability (28).

The bonds of the carbon atoms in a cyclohexane molecule are of two types (Figure 6):

1. Those more or less in the plane of the ring are called equatorial.
2. Those perpendicular to the plane of the ring are called axial. These were formerly designated as polar bonds.

The stability relationship can be inferred by consideration of non-bonded H:H interactions (van der Waals overlap). Since these interactions contribute to instability to an extent which increases with decreasing interatomic distance, this factor can be used to estimate the relative stabilities of different conformations (29). In the boat form the 2,3 and 5,6 groups are closer to each other than in the chair form. Therefore, the chair is the more stable conformation. In addition to van der Waals overlap, electron density in or near axial bonds has been reported to be a factor in steric interactions (30). The interatomic distances between any pair of axial groups are smaller than those between equatorial groups, therefore, axial groups exhibit greater repulsion towards each other. Thus, a compound carrying axial

substituents is less stable than the corresponding compound with equatorial substituents, where non-bonded repulsions are at a minimum.

The generalization that can be made for condensed ring systems made up of six membered rings such as the steroids is that the stable structures are chair conformations with as many as possible of the substituents in the equatorial positions. As a representative structure of the steroid nucleus, the cholestanol molecule (Figure 7) may be examined. In this molecule rings B and C are locked in the chair conformation by trans-fusion to rings A and D. Ring A is free to assume the boat or chair forms. But the instability associated with the boat form of the cyclohexane molecule is augmented by the interaction between the methyl group on C-10 and the hydroxyl group on C-3. Similarly, in cholestane the 3β-hydrogen and the C-10 methyl group oppose the boat form (29).

In the androstane molecule (Figure 8) the ring system of cholestane is present but the side chain at C-17 is lacking. The androstane molecule is described as having three six-membered chair rings and one five-membered ring. The axial hydrogens at position 1, 3, 5, 7, 9 and 14 are in a line parallel to one another. The carbon pairs 2-3, 5-10 and 7-8 lie in one plane and carbon atoms 1, 9, 11, 13 and 14 lie in a second plane parallel to the first (29). Formation of ring D produces a slightly puckered ring due to the bending of the bonds. The hydrogens attached to C-15 and C-16 are eclipsed

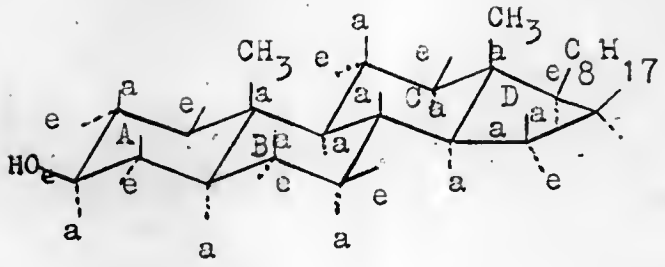

FIGURE 7

Cholestanol.

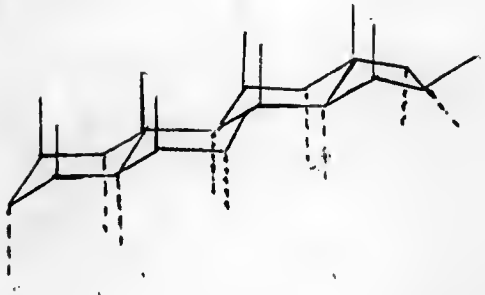

FIGURE 8

5α-Androstane skeleton: α-bonds ---, β-bonds_____.
The equatorial bonds have been omitted for clarity.

and the equatorial and axial concept is not applicable. The bonds at C-15 and C-17 have quasi-axial (a') and quasi-equatorial (e') relationships with respect to ring C.

Useful generalizations regarding stability and reactivity of equatorial and axial substituents have been developed (31-34) and a few of the pertinent ones may be summarized as follows:

1. A substituent is generally more stable in the equatorial than in the axial orientation. Thus, if a substituent can be equilibrated in different orientations, the equatorial will predominate in equilibrium mixtures.
2. An equatorial hydroxyl group is more accessible than the axial and therefore reacts more rapidly than the axial group in esterification or hydrolysis type reactions.
3. In chromatography on paper or on alumina an equatorial alcohol is more strongly adsorbed than its axial isomer.
4. The equatorial or axial orientation of a substituent is often reflected in its infrared absorption spectrum.

Isomerism in Androstanes

Orientation of substituents at specific positions are summarized below (35)

Position	5α-Series		5β-Series	
	α-config	β-config	α-config	β-config
1	a	e	e	a
2	e	a	a	e
3	a	e	e	a
4	e	a	a	e
5	a(AB)	-	-	a(A)e(B)
10	-	a(AB)	-	e(A)a(B)

	5α- and 5β-Series	
6	e	a
7	a	e
8	-	a
9	a	-
11	e	a
12	a	e
13	-	a
14	a	-
15	e'	a'
16	-	-
17	a'	e'

The spatial orientation of a substituent group is designated with reference to the angular methyl groups which are β-oriented. Thus a group projecting in front of the nucleus is referred to as being cis to the methyl groups and a group projecting to the rear is trans to them.

The two major classes of isomeric androstanes are designated as 5α- and 5β-androstane and differ in the spatial configuration of the hydrogen on C-5. In the 5α-configuration the rings A and B trans to each other and in the 5β-series they are cis. Similarly, the 5α-hydrogen is trans to the angular methyl groups and the 5β-hydrogen is cis to them. Substitution of any ring hydrogen further results in the production of stereoisomeric pairs. Thus 5α-androstan-3α-ol and 5α-androstan-3β-ol differ only in the orientation of the hydroxyl group on C-3.

Androstane Isomers Possessing Hormonal Activity

The isolation and structural elucidation of androstanes which exhibit hormonal activity posed a problem to the early investigators. The amounts of steroid hormones which can be isolated from testis or adrenal cortex is very small; however, larger amounts of the steroid hormone or their metabolites are normally found in urine. Functions characterizing the testicular hormone include development of secondary sex characteristics. Among the early methods of bioassay of hormonal activity, the most satisfactory one was the method based on the ability

of the hormone to promote comb growth in capons (36-37). Butenandt (38,39) using urine as the source of the hormone, was the first to isolate and identify one of the principal metabolites of the male hormone. The isolated substance exhibited biological activity. Chemical characterization indicated it to be a sterol-like ketone and Butenandt named the hormone androsterone. The structure of androsterone was further elucidated by partial synthesis from cholesterol by Ruzicka and his associates (40-42). The synthetic compound was identical to that isolated from urine by Butenandt, both chemically and physiologically, and its structure was shown to be 3α-hydroxy-5α-androstan-17-one. Two other biologically active isomers of androstan were synthesized and identified shortly thereafter. These were 3β-hydroxy-5β-androstan-17-one and 3β-hydroxy-5α-androstan-17-one (40-43), both of which have since been isolated from human urine (44,45).

Among the $C_{19}O_3$ androstanes the first to be isolated was 3α, 17β-dihydroxy-5α-androstan-17-one, which was followed by the isolation of 3α-hydroxy-5α-androstane-11,17-dione, 3α-hydroxy-5β-androstane-11,17-dione and 3α,11β-dihydroxy-5β-androstan-17-one (46-48). Androgenic activity was demonstrated for 3α,17β-dihydroxy-5α-androstan-17-one (49).

Human urine also contains steroids having a double bond between carbons 5 and 6. Butendandt and his co-workers (50,51) isolated and identified 3β-hydroxyandrost-5-en-17-one.

Lieberman and his group (52) reported the isolation of 5α-androstane-3,17-dione and 5β-androstane-3,17-dione from normal male and female urines.

During the last decade the complete synthesis of the steroid nucleus and of almost all of the naturally occurring androgens have been accomplished (53-56).

Methods of Assay for Androgens and 17-Ketosteroids

Androgens extracted from urine or tissues were first studied (38,39) by bioassay methods to determine biological activity. By definition only those androstane isomers exhibiting biological activity as measured by comb growth of capons can be called androgens. Urine has been the major source of C_{19} steroids to the investigator and since most of the urinary steroids which exhibit androgenic activity also contain 17-ketone group, the development of chemical methods have been directed toward specific reactions of this group. The procedure most widely used for the determination of 17-ketosteroids is the colorimetric measurement based on the Zimmermann reaction (57). Treatment of a 17-ketone with m-dinitrobenzene in the presence of alkali gives a transient reaction product of violet color but of unknown structure. The colored compound has an absorption maximum at 520 mμ and is sufficiently stable to permit reproducible readings. However, the ketone group on carbon 3 can interfere with the results. Modifications of the above procedure have been reviewed by Zimmermann(58).

Chemical and Physical Methods of Characterization of Steroids

The chemical properties of a steroid like those of any other organic compound should be considered as the reactions of the functional groups of the steroid molecule. It may be assumed that an isolated functional group undergoes reactions with little or no interference from other groups. In cases where two groups are sufficiently close to influence each others properties, their reactions must be considered together.

The relative reactivities of axial and equatorial substituents have already been mentioned. In general, the hydroxyl group on the steroid nucleus will undergo the common reactions known for this group, i.e., esterification, ether formation, epoxide formation, replacement by a halogen, etc. These reactions have been utilized in the isolation and analysis of steroids. In the present investigation two such methods employed were esterification in the preparation of acetyl and trifluoroacetyl derivatives and ether formation in the preparation of trimethylsilyl ethers of the steroids.

Physical methods employed in the characterization of steroids include ultraviolet and infrared absorption spectra, rotatory dispersion, X-ray crystallography and chromatographic analysis.

Ultraviolet absorption was introduced into the steroid field in the early 1930's and became extremely popular as a tool for the detection and determination of trace amounts of compounds with

chromophoric groups. With the accumulation of data, correlation of structure and ultraviolet absorption afforded a powerful new method for the elucidation of the structure of a large variety of steroids. The field has been reviewed by Dorfman (59). More recently Jones and his co-workers (60) investigated the infrared absorption characteristics of steroids. The elucidation of axial and equatorial orientation is in part due to infrared spectral analysis. The variation of optical activity with the wave length of the light was known and used by early investigators until 1860 when Bunsen developed his sodium lamp and a monochromatic light source became available. The application of rotatory dispersion methods to the steroid field was pioneered by Djerassi and his associates (61,62). The absorption bands in rotatory dispersion measurements are sensitive to conformational changes and have been useful in establishing the validity of certain structural relationships. X-ray diffraction pattern of steroids have been useful in supplementing infrared analysis and melting point determinations (63).

With the advent of chromatographic techniques a valuable tool was added to steroid analysis. Adsorption chromatography was applied to the purification and separation of steroids (4). Solvent systems of different polarities were designed for successful separations by partition chromatography (9,11,64). Savard (15,65) investigated the behavior of a wide range of C_{19} and C_{21} ketosteroids and established the applicability of the ligroin-propylene glycol system to the resolution of these ketosteroids. Certain correlations between chromatographic mobility and structure began to emerge. The mobility

of the steroid molecule was observed to decrease with increasing number of oxygen functions. The less than expected retardation in the mobility of a steroid with a C-21 hydroxyl substituent was interpreted as an indication of intramolecular hydrogen bonding. The general pattern of mobilities observed by Savard was consistent with the concept developed by Barton that steroids possessing equatorial hydroxyl groups exhibit lower mobilities than the corresponding isomers with an axial hydroxyl groups (31,66). Furthermore, the correlations between mobility, number and nature of oxygen function, position and orientation of hydroxyl groups together with the relationship of mobilities between 5α- and 5β-isomers provided generalizations which were in agreement with the observations of Lieberman and his group (52,67). From the relationship of chromatographic elution time to structure, certain clues to the nature of unknown compounds were obtained (68). Chromatography before and after acetylation enabled the investigator to assess the number of acetylable hydroxyl groups (69,70).

Nomenclature

Steroid nomenclature is based on the rules defined at the 1950 Ciba Foundation Conference in London (71,72). These rules have been provisionally approved by the International Union of Pure and Applied Chemistry. Since the nomenclature system is of recent origin and since many investigators still employ common names, a brief summary of the approved nomenclature will be given.

According to the new system, the name of the steroid is based on its hydrocarbon nucleus to which prefixes and suffixes are added to indicate the nature of the substituent. The position of these substituents are indicated by the number of the carbon atom to which they are attached. The projections of the substituents to the front and to the rear of the hydrocarbon skeleton are designated as β and α, respectively.

The parent compounds are androstane, pregnane, cholestane, etc,, and with each name, the configuration at C-5 is indicated by the prefix 5α or 5β. Unsaturation is indicated by the suffix 'ene', e.g. 5-ene. The rules for the substituents state that only one kind of substituent in each compound is indicated by a suffix, the remaining substituents are indicated by prefixes. The substituent to be designated by a suffix is chosen according to the following order of priority: carboxylic acid or derivative including esters, carbonyl, alcohol, amine, ether, halogen.

EXPERIMENTAL

Materials and Methods

Compounds Studied

Steroids investigated in the course of this study were chosen for their particular structural features and biological importance. All except two were androstane isomers, the remaining two were progesterone metabolites. The isomeric androstanes employed were:

5α-Series. 5α-androstane, 5α-androstan-3β-ol, 5α-androstan-17β-ol, 5α-androstan-3-one, 5α-androstan-17-one, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5α-androstan-17-one, 17β-hydroxy-5α-androstan-3-one, 17α-hydroxy-5α-androstan-3-one, 5α-androstane-3,17-dione, 3α,11β-dihydroxy-5α-androstan-17-one, 3α-hydroxy-5α-androstane-11,17-dione.

5β-Series. The 5β-isomers of all the compounds listed above were studied simultaneously with the 5α-compounds. The nomenclature is exactly the same as stated above except that the 5β-designation will be substituted for 5α in each instance.

The only unsaturated androstane isomer investigated was 3β-hydroxyandrost-5-en-17-one, which was included because of its biological importance.

The two pregnane isomers were 5β-pregnane-3α,20α-diol and 3α-hydroxy-5β-pregnan-20-one.

In the section of this work involving the separation of 17-ketosteroids, reference will be made to compounds for which common names are still widely used in the literature, especially in the area of biological applications. The common and the approved names of these compounds are summarized below.

Androsterone	3α-Hydroxy-5α-androstan-17-one
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one
11-Ketoetiocholanolone	3α-Hydroxy-5β-androstane-11,17-dione
11β-Hydroxyandrosterone	3α,11β-Dihydroxy-5α-androstan-17-one
Pregnanediol	5β-Pregnane-3α,20α-diol
Pregnanolone	3α-Hydroxy-5β-pregnan-20-one

In the tables summarizing the results obtained in this study, an abbreviated form of naming is used and since one purpose of this investigation was the comparison of 5α/5β isomeric pairs, the orientation at C-5 is written as the prefix in these abbreviated forms. For example, 3α-hydroxy-5α-androstan-17-one is abbreviated as 5α-A-3α-ol,17-one and 3β-hydroxy-5β-androstan-17-one is abbreviated as 5β-A-3β-ol,17-one.

The steroids were obtained from commercial sources and were of sufficiently high purity for direct use. Some, however, showed minute amounts of impurities when examined by gas chromatography. Three of the 17-ketosteroids (3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one and 3β-hydroxyandrost-5-en-17-one) when analyzed by paper chromatography exhibited no impurities.

Preparation of Derivatives

The derivatives of the steroids were prepared by the following procedures.

Acetates. The steroid (2-5 mg) was allowed to react with acetic anhydride (0.5 ml) and pyridine (0.2 ml) at 67° in a dry bath for two hours. The solvents were evaporated under nitrogen and the steroid acetate was dissolved in acetone.

Trifluoroacetates (TFA). The steroid (2-5 mg) was allowed to react with trifluoroacetic anhydride (0.5 ml) and pyridine (0.2 ml). The reaction was complete in a few minutes at room temperature (73). The solvents were evaporated under nitrogen and the derivative was dissolved in acetone.

Trimethylsilyl ethers (TMSi). The steroid (1-3 mg) was allowed to react with hexamethyldisilazane (0.5 ml) and a few drops of trimethylchlorosilane as catalyst for 6-8 hours at room temperature (74). The reaction mixture was centrifuged and the supernatant solvents were evaporated under nitrogen. The residue was triturated with hexane and recentrifuged. The supernatant was again evaporated under nitrogen and the trimethylsilyl ether of the steroid was dissolved in tetrahydrofuran (75).

Studies on the Free Steroids and their Derivatives

The free steroids, acetates and trifluoroacetates were chromatographed from acetone solutions, the trimethylsilyl ethers were chromatographed from tetrahydrofuran solutions. All concentrations were 2 µg/µl. Volumes of 0.2 to 1 µl were used for injections. All measurements were made in duplicate.

One sample of each derivative was analyzed by infrared spectroscopy to ascertain the substitution of the desired group on the steroid nucleus. Some of the derivatives showed small amounts of the free steroid, which were easily identified by their characteristic peaks on the chromatograms.

Reference Standard

Cholestane was used as the reference standard for daily measurements. A saturated solution of cholestane in acetone was injected into the column each day before any series of runs were carried out and was repeated every two or three hours. The retention times of the samples were then related to the retention time of cholestane and were expressed as relative retention times.

Solvents and Reagents

Spectral grade acetone (Fisher Scientific Co.) and reagent grade tetrahydrofuran (Eastman Organic Chemicals), chloroform, pyridine, hexane and acetic anhydride (all from Fisher Scientific Co.) were employed. Pyridine and acetic anhydride were freshly distilled before use. Hexamethyldisilazane, trimethylchlorosilane and trifluoroacetic anhydride were obtained K + K Laboratories and were used without further purification.

Column Supports

Commercially obtained acid-washed (and in the case of the last two, siliconized) diatomaceous earths were used as column supports. These were:

Chromosorb W	(Applied Science Laboratories, State College, Pa.)
Kromat CE	(Burrell Corp., Pittsburg, Pa.)
Anakrom ABS	(Analabs, Hamden, Connecticut)
Diataport S	(F + M Scientific Corp., Avondale, Pa.)

Different mesh sizes employed are indicated under individual column preparations.

Stationary Phase

The following commercially obtained polymers were utilized as the stationary phases:

Silicone Rubber Gums:

SE-30 methyl silicone polymer

SE-52 methyl phenyl silicone polymer

Silicone Fluids:

XF-1150 nitrile silicone polymer (50 mole per cent cyanoethyl)

XF-1105 nitrile silicone polymer (5 mole per cent cyanoethyl)

The above polymers were obtained from General Electric Company, Waterford, New York.

Silicone Copolymer

XE-60 silicone polymer made up of 50% dimethylsiloxane and 50% cyanoethylmethyl siloxane. This was obtained from F + M Scientific Corp., Avondale, Pennslyvania

Fluorinated Silicone Polymer

QF-1-0065 fluoroalkyl silicone polymer.

Silicone Grease

Dow Corning high vacuum silicone grease (DC-silicone) (ethyl acetate extract). The above two were obtained from Dow Corning Corp., Midland, Michigan.

Polyesters

Ethyleneglycol isophtalate (EGIP)

Ethyleneglycol succinate (EGS)

Neopentylglycol adipate (NGAd)

Neopentylglycol sebacate (NGSeb)

These were obtained from Analabs, Hamden, Connecticut.

Hi-Eff 8B - Dimethylcyclohexylsuccinate

This was obtained from Applied Science Laboratories, State College, Pennsylvania.

Bis-(n-phenoxyphenyl)-ether

$(C_6H_5OC_6H_4)_2O$

This compound was obtained from Eastman Organic Chemicals.

Preparation of Columns

The desired amount of liquid phase was weighed and dissolved in a small amount of chloroform (for silicone polymers) or acetone (for all other phases). The solution was transferred, with rinsings, to a beaker containing the weighed support suspended in the same solvent as used for dissolving the liquid polymer. The mixture was heated gently with constant stirring until the solvent had evaporated completely and a free flowing uniformly coated support was obtained.

This method of deposition of the liquid phase onto the support was reliable and reproducible and was preferable to the filtration method (76). In the latter method, a slurry of liquid phase, solvent and support was filtered and then dried with stirring. The concentration of the liquid phase deposited on the support was thus dependent on the mesh size of the support as well as on the volume of solvent used.

The coated support was packed, with gentle tapping, into a copper or stainless steel column one end of which was plugged with glass wool. The open end of the column was flared for ease of packing. A vibrator as an aid in packing was found to be undesirable as it resulted in fragmentation of the support and also produced tightly packed columns. The open end of the column was plugged with glass wool and the column was bent into a U shape, taking care not to constrict the diameter of the tube at the curve. The column was then suspended in the gas chromatograph and cured for 24-48 hours at the desired temperature under 10 psi of pressure of carrier gas. During the curing process the outlet end of the column was not connected in order to prevent the possible accumulation of volatile substances in the detector. After the column was cured, connections to the detector were made and the column was saturated with a 1-3 µg quantity of the reference standard cholestane. The so called 'priming' or saturation process was necessary on those columns where a certain amount of sample bss, attributable to irreversible adsorption was observed. This phenomenon varied from column to column and was dependent on the nature of the sample. For example, on the XE-60 column, there was no observable loss of androstanes while estrogens were adsorbed irreversibly.

Equipment

Measurements were made on two gas chromatographic instruments, Model 600 from Research Specialties Company, Richmond, California, one equipped with a β-ionization detector (77) and the other with a flame

ionization detector (78). Slightly higher sensitivities were observed with the flame ionization unit. It has been reported (79) that multiple hydroxyl groups tend to depress the sensitivity of the β-ionization detector.

Both instruments were equipped with an injection system designed for Tenney and Harris type microdipper injectors (80). In the author's experience this type of injector was more convenient to use and gave more reproducible results than syringe type injectors.

The column temperature was controlled by a proportional controller. A fan inside the heating cabinet was employed to insure uniform distribution of heat throughout the column.

The carrier gases used were argon for the β-ionization detector unit and nitrogen for the flame unit. In the latter unit, compressed air and hydrogen were employed for the flame.

Preliminary Experiments

Results of preliminary experiments will be reported briefly and only for a few representative cases. All measurements were made on the chromatograph equipped with the β-ionization detector unless indicated otherwise. Columns were packed and cured as described. Retention times (t_R) are reported in minutes. All injections were made with a 2 µl aliquots of 1 µg / µl solutions. The β-ionization detector was operated at 1500 volts. Detector temperature = 245-250°. Vaporizer temperature = 290-310°.

SE-30 Methyl Silicone Polymer (SE-30)

Three columns differing in concentration of SE-30, mesh size of support or the length or diameter of the column were employed.

1. A column of 6% SE-30 coated on 30-60 mesh Chromosorb W in a 5-foot 3/16" (o.d.) copper tube was prepared. Variations of retention time with temperature of the column and pressure of the carrier gas are given in Table 1.
2. A column of 3% SE-30 coated on 30-60 mesh Chromosorb W in a 5-foot 3/16" (o.d.) copper tube was prepared.

TABLE 1

Variation of Retention Time[a] with Changes in Temperature and Pressure on 6% SE-30 Column

Conditions		Steroids				
		5α-Androstan-				
Temp. °C	Pres. in psi	3β-ol,17-one	3α-ol,17-one	3-one,17β-ol	3,17-dione	5β-A-3α-ol-17-one
192	11.0	18.6	16.8	17.8	18.5	16.1
210	10.5	10.8	9.8	-	-	-
210	11.0	10.0	9.2	-	-	-
210	11.5	9.4	8.9	-	-	-
210	13.0	8.2	7.7	-	-	-
210	14.0	8.0	7.4	7.6	8.1	7.0
224	15.0	3.8	3.7	3.8	4.0	3.4

[a] In minutes.

Separation factors for androstane isomers under different conditions of temperature and pressure were measured (Table 2). Acetate derivatives of the steroids were prepared with the aim of improving separation factors. Comparative retention time values of free steroids and steroid acetates are given in Table 3. Column conditions: $T = 195^{\circ}$, $P = 18$ psi.

3. A column of 3% SE-30 coated on 50-80 mesh Kromat CE in a 5.5-foot 1/4" (o.d.) copper tube was prepared. Retention values and separation factors of three 17-ketosteroids and the respective acetates are given in Table 4. Column conditions: $T = 200^{\circ}$, $P = 20$ psi.

Individual components could not be resolved when a mixture containing the three principal urinary 17-ketosteroids was chromatographed. Acetylation of the steroids did not result in improved separation. (See sections 2 and 3 above.)

Mixed Phases Containing SE-30

1. SE-30 and DC-silicone

A column coated with 3% SE-30 and 10% DC-silicone was found unsatisfactory. Results were not reproducible. The column was discarded.

TABLE 2

Variation of Separation Factor with Temperature and Pressure on 3% SE-30 Column

Steroids	Conditions		
	224°, 15 psi	210°, 14 psi	192°, 11 psi
5β-A-3α-ol,17-one	1.00	1.00	1.00
5α-A-3α-ol,17-one	1.08	1.06	1.04
5α-A-3β-ol,17-one	1.11	1.14	1.16
5α-A-3-one,17β-ol	1.11	1.09	1.11
5α-A-3,17-dione	1.18	1.16	1.16

TABLE 3

Retention Times[a] on 3% SE-30 Column

Steroids	Free	Acetate
5β-A-3α-ol,17-one	8.9	-
5α-A-3α-ol,17-one	9.2	12.5
5α-A-3-one,17β-ol	9.8	15.9
5β-A-3-one,17β-ol	10.2	14.0
5α-A-3β-ol,17-one	10.1	-
5α-A-3,17-dione	10.4	-
5α-A-3α,17β-diol	10.6	17.5

[a] In minutes.

TABLE 4

Retention Times[a] and Separation Factors on 3% SE-30 Column

Steroids	Free		Acetate	
	t_R	Sep.fac.	t_R	Sep.fac.
5β-A-3α-ol,17-one	6.3	1.00	20.5	1.00
5α-A-3α-ol,17-one	6.5	1.03	20.3	0.99
A-5-en-3β-ol,17-one	7.1	1.14	21.2	1.03

[a] In minutes.

2. SE-30 and Ethyleneglycol Isophtalate (EGIP)

Three columns were prepared:

a. 1.5% SE-30 + 0.5% EGIP

b. 0.5% EGIP

c. 1.5% SE-30

Comparative results obtained on the three columns are given in Table 5, where the individual contributions of each phase is visible, e.g., 2a vs. 2b.

3. SE-30 and Bis-(m-phenoxyphenyl)-ether Mixed Phase

Three columns were prepared:

a. 3% Bis-(m-phenoxyphenyl)-ether (Abbreviated as phenoxy in Table 6.)

b. 3% Bis-(m-phenoxyphenyl)-ether + 3% SE-30 (1:1 ratio)

c. 3% Bis-(m-phenoxyphenyl)-ether + 3% SE-30 (1:2 ratio)

Comparison of the retention times are given in Table 6.

Column conditions: $T = 245^{\circ}$, $P = 30$ psi.

The dependence of the chromatographic pattern of the steroids on the concentration of each component of the stationary phase was noteworthy (3b and 3c above). Separations could not be affected due to large peak areas and some skewing of the peaks. After a short period of use, irregular bizarre patterns indicative of phase decomposition were observed on this column and further work was not continued.

TABLE 5

Comparison of Separation Factors on SE-30 and Ethyleneglycol Isophtalate (EGIP) Columns

FREE	1.5% SE-30 + 0.5% EGIP	0.5% EGIP	1.5% SE-30
5β-A-3α-ol,17-one	1.03	1.00	0.70
5α-A-3α-ol,17-one[a]	1.00	1.00	1.00
5β-A-3-one,17β-ol	1.33	1.12	1.30
5α-A-3-one,17β-ol	1.18	1.12	0.87
5α-A-3β-ol,17-one	1.17	1.03	0.83
A-5-en-3β-ol,17-one	1.08	-	0.87
5α-A-3,17-dione	1.36	1.32	0.80
5α-A-3α,17β-diol	1.10	1.10	0.87
ACETATES			
5β-A-3α-ol,17-one	1.06	1.17	1.25
5α-A-3α-ol,17-one[a]	1.00	1.00	1.00
5β-A-3-one,17β-ol	1.34	1.75	1.25
5α-A-3-one,17β-ol	1.57	2.00	1.05
A-5-en-3β-ol,17-one	1.43	-	1.40
5α-A-3α,17β-diol	1.20	0.96	1.20

[a] The values of these compounds were used as the point of reference for the calculation of separation factors of the free steroids and the acetates, respectively.

TABLE 6

Comparison of Retention Times[a] on SE-30 and Bis-(m-phenoxyphenyl)-ether Columns

Steroid Acetates	3% Phenoxy		3% SE-30 and 3% Phenoxy (1:1 ratio)		3% SE-30 and 3% Phenoxy (2:1 ratio)	
	t_R	Sep.fac.	t_R	Sep.fac.	t_R	Sep.fac.
5α-A-3α-ol,17-one	17.6	4.4	21.5	1.36	12.5	0.54
5β-A-3α-ol,17-one	12.0	3.0	23.0	1.45	15.8	0.69
A-5-en-3β-ol,17-one	8.0	2.0	22.5	1.43	14.0	0.61
Cholestane	4.0	1.0	15.8	1.00	23.0	1.00

[a] In minutes.

SE-52 Methyl Phenyl Silicone Polymer (SE-52)

A column of 3% SE-52 coated on 100-110 mesh Anakrom ABS in a 4-foot 3/16" (o.d.) copper tube was prepared. Retention time values are given in Table 7. Column conditions: T = 212°, P = 25 psi and flow rate = 2 ml/min.

There was no observable advantage to be gained by the use of SE-52 polymer as compared to SE-30 phase. The fine mesh support caused a considerable decrease in flow rate and it was decided to perform subsequent measurements on coarser than 100 mesh supports.

Dow Corning High Vacuum Silicone Grease

A column of 30% DC-silicone coated on 40-50 mesh Anakrom ABS in a 5-foot 3/16" (o.d.) copper tube was prepared. Column was too retentive even after it was cut down to a length of 3 feet.

Ethyleneglycol Succinate Polyester (EGS)

A column of 12% EGS coated on 30-60 mesh Chromosorb W in 2.5-foot 3/16" (o.d.) copper column was prepared. Column was very retentive; no peaks were observed.

Neopentylglycol Adipate Polyester (NGAd)

A column of 3% NGAd coated on 30-60 mesh Chromosorb W in a 4-foot, 3/16" (o.d.) copper tube was prepared. The column required

TABLE 7

Retention Times[a] on 3% SE-52 Column

Steroid	t_R	Rel. t_R
Cholestane	14.6	1.00
5β-A-17-one	2.9	0.20
5β-A-3-one,17β-ol	6.7	0.46
5β-A-3α,17β-diol	6.2	0.42
5α-A-3α,17β-diol	6.5	0.44
5α-A-3β,17β-diol	6.7	0.46
5β-A-3,17-dione	6.7	0.46

[a] In minutes.

daily priming with cholestane. Isomeric retention times are given in Table 8. Column conditions: T = 220°, P = 30 psi, flow rate = 108 ml/min.

The retention of solutes on the 4-foot NGAd column was very low. Therefore, a column of 3% NGAd coated on 30-60 mesh Chromosorb W in a 6-foot 3/16" (o.d.) copper tube was prepared. The retention data on three 17-ketosteroid acetates are reported in Table 9. Column conditions: T = 230°, P = 20 psi, flow rate = 54 ml/min.

Neopentylglycol Sebacate Polyester (NGSeb)

A column of 3% NGSeb coated on 30-60 mesh Chromosorb W in a 6-foot 3/16" (o.d.) copper tube was prepared. The results appeared satisfactory but the column was subjected to high temperatures overnight by a breakdown in the temperature control unit. It was discarded.

Silicone Nitrile Fluids XF-1150 and XF-1105

A column of 3% XF-1150 coated on 30-60 mesh Kromat CE in a 6-foot 3/16" (o.d.) copper tube was prepared. The flow rate was extremely slow and loss of phase was detected. The column was discarded.

TABLE 8

Isomeric Retention Times[a] on 3% Neopentylglycol Adipate Column

Steroid	t_R	Rel. t_R
5β-A-3,17-dione	5.8	3.06
5α-A-3,17-dione	6.5	3.32
5α-A-3β,17β-diol	7.7	4.05
5α-A-3α,17β-diol	6.7	3.53
5α-A-3α,17β-diol	6.7	3.53
5β-A-3α,17β-diol	6.7	3.53
5α-A-3α-ol,17-one	5.4	2.84
5β-A-3α-ol,17-one	5.5	2.89
5α-A-3α-ol,17-one	5.4	2.84
5α-A-3β-ol,17-one	6.1	3.21
A-5-en-3β-ol,17-one	6.4	3.37
5α-A-3β-ol,17-one	6.1	3.21
5β-A-3,17-dione	5.8	3.06
5β-A-3-one,17β-ol	6.7	3.53
5β-A-3-one,17β-ol	6.7	3.53
5β-A-3α-ol,17β-diol	6.7	3.53
5α-A-17-ol	1.0	0.53
Cholestane	1.9	1.00

[a] In minutes.

TABLE 9

Retention Times[a] of 17-Ketosteroid Acetates on
3% Neopentylglycol Adipate Column

Steroid Acetates	t_R	Rel.t_R
5α-A-3α-ol,17-one	23.5	1.6
5β-A-3α-ol,17-one	25.0	1.7
A-5-en-3β-ol,17-one	27.5	1.8
Cholestane	15.0	1.0

[a] In minutes.

A column of 3% XF-1105 coated on 50-60 mesh Kromat CE in a 6-foot 3/16" (o.d.) copper tube was prepared. Retention data and separation factors are given in Table 10. Column conditions: T = 250°, P = 35 psi, flow rate = 20 ml/min. The flow rate was maximal.

Even though the separation factors indicated theoretical separations, actual resolutions of the components of the mixture were not accomplished. The wide peak area, presence of some skewing and the high retentivity of the column in spite of the high temperature of operation were major drawbacks.

QF-1-0065 Fluoroaklyl Polymer (QF-1-0065)

A column of 3% QF-1-0065 coated on 70-80 mesh Anakrom in a 6-foot 1/8" (o.d.) stainless steel tube was prepared. Measurements were made on the chromatograph equipped with the flame ionization detector. Column conditions: T = 238°, P = 14 psi. It was hoped that selective retention of this column for ketones could be used to advantage in effecting separations. Loss of phase and irregular chromatographic patterns were observed and in spite of numerous efforts, no reliable data could be obtained. One interpretation of the data was that the phase had reacted with the glass wool plug.

TABLE 10

Retention Times[a] and Separation Factors on 3% XF-1105 Column

Steroid	t_R	Rel. t_R	Sep.fac.
5β-A-3α-ol,17-one	13.8	0.63	1.00
5α-A-3α-ol,17-one	16.8	0.76	1.22
A-5-en-3β-ol,17-one	24.0	1.09	1.74
5β-A-3α-ol,11,17-dione	25.0	1.14	1.81
5α-A-3α,11β-diol,17-one	37.8	1.72	0.74
Cholestane	22.0	1.00	1.59

[a] In minutes.

Discussion of Preliminary Experiments

The choice of the proper solvent for the stationary phase was the first consideration for this study. The extended ring system of the steroid molecule provides a fairly wide area of hydrocarbon skeleton for interactions with solvent molecules. Because of the relative rigidity of the skeleton and the short range effectiveness of the van der Waals forces of attraction between the solute and the solvent molecules, these interactions are sensitive to steric factors (3). The interactions between the parts of the solute and the solvent molecules compete with interactions between the solvent molecules themselves. The solution of a polar substance in a polar solvent may therefore be considered to depend upon the ability of the polar groups of the solute to attract parts of the solvent molecule with a force comparable to or greater than that among the solvent molecules themselves. A non-polar solute, which cannot exert such a force of attraction is squeezed out of the polar solvent and the normal attractive forces in the pure solvent are re-established. Non-polar solvents, on the other hand, exhibit far less short range attractive forces than do polar solvents. The molecules of non-pplar solvents are attracted to one another by relatively weak dispersion forces and a non-polar solute may exhibit the same weak attractions as do the solvent molecules themselves. A polar solute introduced into a non-polar solvent is expected to have very little or no interaction with the solvent.

In highly unsaturated molecules, stronger interactions may occur due to delocalization of electrons and result in an increase in the dispersion forces (81). The theoretical basis of such interactions is still incomplete, but empirically it is known that unsaturated molecules tend to attract one another more strongly than they attract saturated molecules. Similarly, unsaturated groups attract the molecules of polar substances much more strongly than do saturated groups. Unsaturated groups may thus be assumed to be polarizable.

In the present investigation the liquid solvent phases selected, in addition to the chemical property considerations mentioned above, had to meet as many of the following requirements as possible at the temperature of operations: low volatility, thermal stability, low viscosity and chemical inertness. For temperatures above 200°, as employed in this study, the choice of phases was limited. Satisfactory phases at these higher temperatures included silicone gums and polyesters of low volatility.

Since oxygen substituted steroids of relatively high polarity were to be studied simultaneously with their derivatives of higher or lower polarity two basic types of liquid phases were indicated. A polar stationary phase would enhance the interaction of the polar groups of the steroid molecule with the phase and a non-polar phase would be expected preferrentially to attract the non-polar functions.

SE-30 polymer was found to be a satisfactory non-selective phase. Bis-(m-phenoxyphenyl)-ether was interesting in the unique retention pattern of the 17-ketosteroid acetates on this stationary phase (Table 6). However the compound was not stable at elevated temperatures. The polyester phases exhibited the desired polarity but the choice was narrowed to those which were thermostable. The separation factors of the androstane isomers on XF-1105 phase was promising, but here again a phase combining the selective properties of the silicone nitrile fluids and the thermal stability of silicone polymers was indicated.

After preliminary investigations with different silicone elastermers, silicone nitrile polymers and various polyester phases, both singly and in mixtures, four stationary phases which differed in degrees of polarity and selectivity were chosen: the methyl substituted silicone gum SE-30 (non-selective, heat stable), the silicone nitrile polymer XE-60 (similar to silicone nitrile fluids but more stable at elevated temperatures) and neopentylglycol sebacate and Hi-Eff 8B (heat stable, polar) polyester phases.

The effect of varying the chemical composition of the stationary phase for the separation of a wide range of steroids had been reported (82-86). Lipsky and Landowne (83) had reported the thermal stability and polar properties of neopentylglycol sebacate. SE-30 silicone polymer had found wide use as stationary phase since the early applications of the gas-liquid chromatographic methods to steroid analyses.

XE-60 and Hi-Eff 8B were two new polymers developed specifically for high temperature uses.

Comparative Experiments

All of the androstanes and the derivatives listed under Materials and Methods were utilized in this portion of the study. The concentration of the solutions were 2 µg/µl and 0.2 to 1 µl aliquots were injected. The detector and vaporizer temperatures were the same as those given in the preliminary experiments. The gas chromatograph equipped with the β-ionization detector (operated at 1500 volts) was employed unless indicated otherwise.

Following columns were used:

SE-30. A column of 3% SE-30 coated on 40-50 mesh Anakrom ABS in a 5-foot 3/16" (o.d.) copper tube was prepared. The chromatograph equipped with the flame ionization detector was employed. Column conditions : T = 260°, P = 10 psi. Retention time for cholestane = 12-14 minutes.

XE-60. A column of 3% XE-60 coated on 80-100 mesh Diatoport S in a 6-foot 1/8" (o.d.) stainless steel tube was prepared. The chromatograph equipped with the flame ionization detector was employed. Column conditions: T = 260-262°, P = 14 psi. Retention time of cholestane = 6-9 minutes.

Hi-Eff 8B. A column of 3% Hi-Eff 8B coated on 40-50 mesh Anakrom ABS in a 5-foot 3/16" (o.d.) copper tube was prepared. Column conditions: T = 238°, P = 15 psi and flow rate = 39 ml. Retention time for cholestane = 6-8 minutes.

A column of 1% Hi-Eff 8B coated on 80-100 mesh Diataport S in a 6-foot 1/8" (o.d.) stainless steel tube was prepared. Column conditions: T = 242°, P = 33 psi and flow rate = 24 ml/min. Retention time for cholestane = 5-7 minutes.

NGSeb. A column of 3% NGSeb on 30-60 mesh Chromosorb W in a 6-foot 1/8" (o.d.) stainless steel tube was prepared. Column conditions: T = 232°, P = 25 psi and flow rate = 74 ml/min. Retention time for cholestane = 8-9 minutes for the measurements of the free steroids and the steroid acetates and 11 minutes for all other compounds.

A column of 1% NGSeb coated on 80-90 mesh Anakrom ABS in a 6-foot 1/8" (o.d.) stainless steel tube was prepared. Column conditions: T = 231°, P = 30 psi and flow rate = 24 ml/min. Retention time for cholestane = 10 minutes.

The relative retention time values with respect to cholestane observed on the six columns under the conditions described above are summarized in Table 11.

TABLE 11 - SE-30 3%[a]

A Comparison of the Relative Retention Times of Substituted Androstanes

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane	0.21	-	-	-
5β-Androstane	0.20	-	-	-
5α-A-17β-ol	0.32	0.39	0.33	-
5β-A-17β-ol	0.41	0.52	0.49	-
5α-A-17-one	0.32	-	-	-
5β-A-17-one	0.42	-	-	-
5α-A-3β-ol	0.31	0.39	0.34	0.24
5β-A-3β-ol	0.29	0.35	0.31	0.22
5α-A-3-one	0.33	-	-	-
5β-A-3-one	0.32	-	-	-
5α-A-3α,17β-diol	0.54	0.89	0.58	0.28
5β-A-3α,17β-diol	0.51	0.87	0.61	0.32
5α-A-3β,17β-diol	0.59	0.98	0.69	0.36
5β-A-3β,17β-diol	0.49	0.86	0.56	-
5α-A-3α-ol,17-one	0.51	0.58	0.47	-
5β-A-3α-ol,17-one	0.47	0.56	0.46	0.28
5α-A-3β-ol,17-one	0.55	0.66	0.55	0.30
5β-A-3β-ol,17-one	-	-	-	-
5α-A-3-one,17β-ol	0.59	0.76	0.64	0.35
5β-A-3-one,17β-ol	0.54	0.69	0.59	0.33
5α-A-3-one,17α-ol	0.56	0.75	0.60	-
5β-A-3-one,17α-ol	0.53	0.61	0.47	0.33
5α-A-3,17-dione	0.58	-	-	-
5β-A-3,17-dione	0.50	-	-	-
A-5-en-3β-ol,17-one	0.61	0.64	0.55	0.33

TABLE 11 - XE-60 3%[b]
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane	0.27	-	-	-
5β-Androstane	0.25	-	-	-
5α-A-17β-ol	0.60	0.71	0.38	-
5β-A-17β-ol	0.89	1.04	0.52	-
5α-A-17-one	0.65	-	-	-
5β-A-17-one	0.95	-	-	-
5α-A-3β-ol	0.65	0.70	0.39	-
5β-A-3β-ol	0.53	0.59	0.32	-
5α-A-3-one	0.77	-	-	-
5β-A-3-one	0.68	-	-	-
5α-A-3α,17β-diol	1.67	2.05	0.49	-
5β-A-3α,17β-diol	1.68	1.95	0.50	-
5α-A-3β,17β-diol	1.89	2.21	0.66	-
5β-A-3β,17β-diol	1.56	1.90	0.51	-
5α-A-3α-ol,17-one	2.00	2.14	0.87	-
5β-A-3α-ol,17-one	1.90	2.15	0.94	-
5α-A-3β-ol,17-one	2.10	2.44	1.16	-
5β-A-3β-ol,17-one	1.98	2.98	0.86	-
5α-A-3-one,17β-ol	2.58	2.81	1.32	-
5β-A-3-one,17β-ol	2.44	2.53	1.18	-
5α-A-3-one,17α-ol	2.03	2.32	1.16	-
5β-A-3-one,17α-ol	2.14	2.14	0.91	-
5α-A-3,17-dione	2.64	-	-	-
5β-A-3,17-dione	2.48	-	-	-
A-5-en-3β-ol,17-one	2.01	2.38	1.10	-

TABLE 11 - Hi-Eff 3%[c]
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane	0.13	-	-	-
5β-Androstane	0.12	-	-	-
5α-A-17β-ol	0.71	0.58	0.22	0.16
5β-A-17β-ol	1.48	1.27	-	-
5α-A-17-one	0.61	-	-	-
5β-A-17-one	1.15	-	-	-
5α-A-3β-ol	0.75	0.71	0.24	0.20
5β-A-3β-ol	0.61	0.57	-	0.15
5α-A-3-one	0.73	-	-	-
5β-A-3-one	0.67	-	-	-
5α-A-3α,17β-diol	3.94	2.48	0.28	0.27
5β-A-3α,17β-diol	3.85	2.82	0.37	0.30
5α-A-3β,17β-diol	4.19	3.18	0.42	0.24
5β-A-3β,17β-diol	3.50	2.42	0.28	0.23
5α-A-3α-ol,17-one	3.73	2.62	0.74	0.80
5β-A-3α-ol,17-one	3.58	2.86	1.03	0.61
5α-A-3β-ol,17-one	3.97	3.44	1.20	0.67
5β-A-3β-ol,17-one	-	-	-	-
5α-A-3-one,17β-ol	4.61	3.88	1.27	1.24
5β-A-3-one,17β-ol	4.18	3.44	1.13	1.14
5α-A-3-one,17α-ol	4.36	3.31	1.09	-
5β-A-3-one,17α-ol	3.97	2.91	0.81	0.78
5α-A-3,17-dione	4.00	-	-	-
5β-A-3,17-dione	3.64	-	-	-
A-5-en-3β-ol,17-one	4.00	3.38	1.16	1.02

TABLE 11 - Hi-Eff 1%[d]
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane	0.27	-	-	-
5β-Androstane	0.26	-	-	-
5α-A-17β-ol	0.74	0.65	0.29	0.16
5β-A-17β-ol	1.32	1.04	0.41	-
5α-A-17-one	0.63	-	-	-
5β-A-17-one	1.14	-	-	-
5α-A-3β-ol	0.75	0.67	0.32	0.20
5β-A-3β-ol	0.63	0.57	0.25	0.15
5α-A-3-one	0.78	-	-	-
5β-A-3-one	0.70	-	-	-
5α-A-3α,17β-diol	3.23	3.27	0.88	0.27
5β-A-3α,17β-diol	3.20	2.52	1.07	0.30
5α-A-3β,17β-diol	3.60	2.86	1.18	0.24
5β-A-3β,17β-diol	2.93	2.23	0.86	0.23
5α-A-3α-ol,17-one	2.88	2.43	0.78	0.80
5β-A-3α-ol,17-one	2.92	2.61	0.99	0.61
5α-A-3β-ol,17-one	3.24	2.95	1.15	0.67
5β-A-3β-ol,17-one	-	-	-	-
5α-A-3-one,17β-ol	3.84	3.28	1.24	1.24
5β-A-3-one,17β-ol	3.49	3.04	1.13	1.14
5α-A-3-one,17α-ol	3.18	2.92	1.11	-
5β-A-3-one,17α-ol	3.26	2.58	0.85	0.78
5α-A-3,17-dione	3.63	-	-	-
5β-A-3,17-dione	3.32	-	-	-
A-5-en-3β-ol,17-one	3.32	2.92	1.14	1.02

TABLE 11 - NGSeb 3%[e]
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane	0.09	-	-	-
5β-Androstane	0.08	-	-	-
5α-A-17β-ol	0.47	0.44	0.20	0.16
5β-A-17β-ol	0.84	0.80	-	0.32
5α-A-17-one	0.39	-	-	-
5β-A-17-one	0.71	-	-	-
5α-A-3β-ol	0.47	0.47	0.21	0.18
5β-A-3β-ol	0.38	0.37	-	0.13
5α-A-3-one	0.49	-	-	-
5β-A-3-one	0.42	-	-	-
5α-A-3α,17β-diol	2.27	1.69	0.31	0.29
5β-A-3α,17β-diol	2.08	1.89	0.35	0.34
5α-A-3β,17β-diol	2.44	2.20	0.44	0.41
5β-A-3β,17β-diol	1.93	1.66	0.28	0.28
5α-A-3α-ol,17-one	1.83	1.61	0.58	0.67
5β-A-3α-ol,17-one	1.80	1.76	0.72	0.73
5α-A-3β-ol,17-one	2.10	2.00	0.91	0.94
5β-A-3β-ol,17-one	1.88	-	-	-
5α-A-3-one,17β-ol	2.61	2.38	1.07	1.18
5β-A-3-one,17β-ol	2.20	1.98	0.91	0.97
5α-A-3-one,17α-ol	2.22	2.12	0.94	-
5β-A-3-one,17α-ol	2.05	1.76	0.62	0.50
5α-A-3,17-dione	2.27	-	-	-
5β-A-3,17-dione	1.98	-	-	-
A-5-en-3β-ol,17-one	2.09	1.99	0.90	0.84

TABLE 11 - NGSeb 1%[f]
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane	0.14	-	-	-
5β-Androstane	0.12	-	-	-
5α-A-17β-ol	0.46	0.45	0.24	-
5β-A-17β-ol	0.80	0.80	0.39	-
5α-A-17-one	0.39	-	-	-
5β-A-17-one	0.68	-	-	-
5α-A-3β-ol	0.48	0.49	0.26	-
5β-A-3β-ol	0.39	0.41	0.40	-
5α-A-3-one	0.48	-	-	-
5β-A-3-one	0.42	-	-	-
5α-A-3α,17β-diol	1.88	0.73	0.36	-
5β-A-3α,17β-diol	1.82	0.84	0.40	-
5α-A-3β,17β-diol	2.14	0.82	0.50	-
5β-A-3β,17β-diol	1.70	0.65	0.35	-
5α-A-3α-ol,17-one	1.64	1.50	0.60	-
5β-A-3α-ol,17-one	1.59	1.57	0.73	-
5α-A-3β-ol,17-one	1.85	1.88	0.88	-
5β-A-3β-ol,17-one	1.53	1.48	0.74	-
5α-A-3-one,17β-ol	2.24	2.10	0.96	-
5β-A-3-one,17β-ol	1.96	1.91	0.87	-
5α-A-3-one,17α-ol	-	-	-	-
5β-A-3-one,17α-ol	1.82	1.61	0.63	-
5α-A-3,17-dione	2.00	-	-	-
5β-A-3,17-dione	1.78	-	-	-
A-5-en-3β-ol,17-one	1.86	1.78	0.84	

Retention time of Cholestane: [a] 12-14, [b] 6-9, [c] 6-8, [e] 9-10, [f] 10 minutes.

Elution pattern of the isomeric free steroids on the six columns are given in Table 12.

The calculated group retention factors for the hydroxyl, ketone, acetyl and trimethylsilyl groups are given in Table 13. A comparison of observed and calculated retention time values based on average log k values are reported in Table 14.

The changes in retention time upon derivative formation are calculated according to the equation.

$$(r_{\text{derivative}} / r_{\text{free steroid}}) \times 100 = r' \%$$

The results are summarized in Table 15.

The average change in retention time consequent to derivative formation and the dependence of this change upon the number of substituent groups are shown in Table 16.

Table 17 gives the values of the T term calculated according to the following equation

$$T = \frac{t'_{XE\text{-}60} - t'_{SE\text{-}30}}{t'_{SE\text{-}30}}$$

A comparison of the ketone selective properties of the four column phases is given in Table 18.

TABLE 12 - SE-30 3%

Comparison of Retention Data of
Isomeric Pairs: Substituent Effects

Functional Group	5α	5β
No Substitution	0.21	0.20
17β-ol	0.32	0.41
3β-ol	0.31	0.29
3α,17β-diol	0.54	0.51
3β,17β-diol	0.59	0.49
17-one	0.32	0.42
3-one	0.33	0.32
3,17-dione	0.58	0.50
3α-ol,17-one	0.51	0.47
3β-ol,17-one	0.55	-
3-one,17β-ol	0.59	0.54
3-one,17α-ol	0.56	0.53

TABLE 12 - XE-60 3%
(cont.)

Functional Group	5α	5β
No Substitution	0.27	0.25
17β-ol	0.60	0.89
3β-ol	0.65	0.53
3α,17β-diol	1.67	1.68
3β,17β-diol	1.89	1.56
17-one	0.65	0.95
3-one	0.77	0.68
3,17-dione	2.64	2.48
3α-ol,17-one	2.00	1.90
3β-ol,17-one	2.10	1.98
3-one,17β-ol	2.58	2.44
3-one,17α-ol	2.09	2.14

TABLE 12 - Hi-Eff 3%
(cont.)

Functional Group	5α	5β
No Substitution	0.13	0.12
17β-ol	0.71	1.48
3β-ol	0.75	0.61
3α,17β-diol	3.94	3.85
3β,17β-diol	4.19	3.18
17-one	0.61	1.15
3-one	0.73	0.67
3,17-dione	4.00	3.64
3α-ol,17-one	3.73	3.58
3β-ol,17-one	3.97	-
3-one,17β-ol	4.61	4.18
3-one,17α-ol	4.36	3.97

TABLE 12 - Hi-Eff 1%
(cont.)

Functional Group	5α	5β
No Substitution	0.27	0.26
17β-ol	0.74	1.32
3β-ol	0.75	0.63
3α,17β-diol	3.23	3.20
3β,17β-diol	3.60	2.93
17-one	0.63	1.14
3-one	0.78	0.70
3,17-dione	3.63	3.32
3α-ol,17-one	2.88	2.92
3β-ol,17-one	3.24	-
3-one,17β-ol	3.84	3.49
3-one,17α-ol	3.18	3.26

TABLE 12 - NGSeb 3%
(cont.)

Functional Group	5α	5β
No Substitution	0.09	0.08
17β-ol	0.47	0.84
3β-ol	0.47	0.38
3α,17β-diol	2.27	2.08
3β,17β-diol	2.44	1.93
17-one	0.39	0.71
3-one	0.49	0.42
3,17-dione	2.27	1.98
3α-ol,17-one	1.83	1.80
3β-ol,17-one	2.10	1.88
3-one,17β-ol	2.61	2.20
3-one,17α-ol	2.22	2.05

TABLE 12 - NGSeb 1%
(cont.)

Functional Group	5α	5β
No Substitution	0.13	0.12
17β-ol	0.46	0.80
3β-ol	0.48	0.39
3α,17β-diol	1.88	1.82
3β,17β-diol	2.14	1.70
17-one	0.39	0.68
3-one	0.48	0.42
3,17-dione	2.00	1.78
3α-ol,17-one	1.64	1.59
3β-ol,17-one	1.85	1.53
3-one,17β-ol	2.25	1.96
3-one,17α-ol	-	1.82

TABLE 13 - SE-30 3%

Calculated Group Retention Factors
($\log r = \log r_n + \log k_a + \log k_b$)

Functional Group	5α			5β		
	Free	Acet	TMSi	Free	Acet	TMSi
17β-ol						
17β-ol / Androstane	0.19	0.28	0.20	0.31	0.41	
3β,17β-diol / 3β-ol	0.27	0.40	0.31	0.23	0.39	
3-one,17β-ol / 3-one	0.25	0.36	0.29	0.23	0.34	
17α-ol						
3-one,17α-ol / 3-one	0.23	0.36	0.26	0.22	0.28	
3β-ol						
3β-ol / Androstane	0.18	0.28	0.22	0.16	0.25	
3β,17β-diol / 17β-ol	0.26	0.40	0.32	0.08	0.22	
3β-ol,17-one / 17-one	0.24	0.31	0.23	-	-	
3α-ol						
3α,17β-diol / 17β-ol	0.22	0.35	0.25	0.10	0.23	
3α-ol,17-one / 17-one	0.21	0.26	0.17	0.05	0.13	
17-one						
17-one / Androstane	0.19	-	-	0.32	0.32	
3β-ol,17-one / 3β-ol	0.25	0.22	0.20	-	-	
3,17-dione / 3-one	0.24	-	-	0.20	-	
3-one						
3-one / Androstane	0.20	-	-	0.20	-	
3-one,17β-ol / 17β-ol	0.26	0.29	0.29	0.12	0.13	
3,17-dione / 17-one	0.26	-	-	0.08	-	

TABLE 13 - XE-60 3%
(cont.)

Functional Group	5α			5β		
	Free	Acet	TMSi	Free	Acet	TMSi
17β-ol						
17β-ol / Androstane	0.35	0.42	0.14	0.55	0.62	0.32
3β,17β-diol / 3β-ol	0.41	0.50	0.23	0.47	0.51	0.20
3-one,17β-ol / 3-one	0.51	0.63	0.23	0.55	0.57	0.24
17α-ol						
3-one,17α-ol / 3-one	0.42	0.48	0.18	0.50	0.50	0.13
3β-ol						
3β-ol / Androstane	0.38	0.41	0.16	0.33	0.37	0.11
3β,17β-diol / 17β-ol	0.45	0.49	0.25	0.24	0.26	-0.01
3β-ol,17-one / 17-one	0.51	0.57	0.25	0.32	0.50	-0.04
3α-ol						
3α,17β-diol / 17β-ol	0.44	0.46	0.12	0.28	0.27	-0.02
3α-ol,17-one / 17-one	0.49	0.47	0.07	0.30	0.35	0.00
17-one						
17-one / Androstane	0.38	-	-	0.58	-	-
3β-ol,17-one / 3β-ol	0.51	0.54	0.47	0.57	0.70	0.43
3,17-dione / 3-one	0.53	-	-	0.56	-	-
3-one						
3-one / Androstane	0.45	-	-	0.43	-	-
3-one,17β-ol / 17β-ol	0.62	0.60	0.55	0.44	0.38	0.36
3,17-dione / 17-one	0.61	-	-	0.42	-	-

TABLE 13 - Hi-Eff 3%
(cont.)

Functional Group	5α			5β		
	Free	Acet	TMSi	Free	Acet	TMSi
17β-ol						
17β-ol / Androstane	0.74	0.66	0.23	1.09	1.02	-
3β,17β-diol / 3β-ol	0.75	0.65	0.26	0.76	0.63	-
3-one,17β-ol / 3-one	0.80	0.73	0.24	0.79	0.71	0.23
17α-ol						
3-one,17α-ol / 3-one	0.78	0.66	0.18	0.77	0.64	0.08
3β-ol						
3β-ol / Androstane	0.77	0.74	0.27	0.70	0.67	-
3β,17β-diol / 17β-ol	0.77	0.74	0.28	0.37	0.28	-
3β-ol,17-one / 17-one	0.81	0.75	-0.70	-	-	-
3α-ol						
3α,17β-diol / 17β-ol	0.74	0.63	0.10	0.41	0.35	-
3α-ol,17-one / 17-one	0.79	0.63	0.08	0.49	0.40	-0.05
17-one						
17-one / Androstane	0.68	-	-	0.98	-	-
3β-ol,17-one / 3β-ol	0.72	0.68	-0.30	-	-	-
3,17-dione / 3-one	0.74	-	-	0.73	-	-
3-one						
3-one / Androstane	0.76	-	-	0.74	-	-
3-one,17β-ol / 17β-ol	0.81	0.82	0.76	0.45	0.43	-
3,17-dione / 17-one	0.82	-	-	0.50	-	-

TABLE 13 - Hi-Eff 1%
(cont.)

Functional Group	5α			5β		
	Free	Acet	TMSi	Free	Acet	TMSi
17β-ol						
17β-ol / Androstane	0.44	0.38	0.03	0.71	0.61	0.20
3β,17β-diol / 3β-ol	0.68	0.63	0.57	0.67	0.59	0.54
3-one,17β-ol / 3-one	0.69	0.62	0.20	0.70	0.64	0.21
17α-ol						
3-one,17α-ol / 3-one	0.61	0.57	0.15	0.67	0.57	0.08
3β-ol						
3β-ol / Androstane	0.45	0.40	0.08	0.39	0.35	-0.01
3β,17β-diol / 17β-ol	0.69	0.64	0.61	0.35	0.33	0.45
3β-ol,17-one / 17-one	0.71	0.67	0.26	0.48	0.50	-0.18
3α-ol						
3α,17β-diol / 17β-ol	0.64	0.54	0.48	0.38	0.38	0.42
3α-ol,17-one / 17-one	0.66	0.59	0.09	0.40	0.36	-0.06
17-one						
17-one / Androstane	0.37	-	-	0.65	-	-
3β-ol,17-one / 3β-ol	0.64	0.64	0.56	0.74	0.80	0.48
3,17-dione / 3-one	0.67	-	-	0.67	-	-
3-one						
3-one / Androstane	0.46	-	-	0.43	-	-
3-one,17β-ol / 17β-ol	0.71	0.70	0.63	0.42	0.47	0.44
3,17-dione / 17-one	0.76	-	-	0.46	-	-

TABLE 13-NGSeb 3%
(/cont.)

Functional Group	5α			5β		
	Free	Acet	TMSi	Free	Acet	TMSi
17β-ol						
17β-ol / Androstane	0.72	0.69	0.35	1.02	1.00	--
3β,17β-diol / 3β-ol	0.71	0.67	0.32	0.71	0.65	--
3-one,17β-ol / 3-one	0.73	0.69	0.34	0.72	0.67	0.34
17α-ol						
3-one,17α-ol / 3-one	0.66	0.64	0.28	0.69	0.62	0.17
3β-ol						
3β-ol / Androstane	0.72	0.72	0.37	0.68	0.66	--
3β,17β-diol / 17β-ol	0.71	0.70	0.34	0.36	0.32	--
3β-ol,17-one / 17-one	0.73	0.71	0.37	0.42	--	--
3α-ol						
3α,17β-diol / 17β-ol	0.68	0.58	0.19	0.39	0.37	--
3α-ol,17-one / 17-one	0.67	0.62	0.17	0.40	0.39	0
17-one						
17-one / Androstane	0.64	--	--	0.94	--	--
3β-ol,17-one / 3β-ol	0.65	0.63	0.64	0.69	--	--
3,17-dione / 3-one	0.67	--	--	0.67	--	--
3-one						
3-one / Androstane	0.73	--	--	0.72	--	--
3-one,17β-ol / 17β-ol	0.74	0.73	0.73	0.42	0.39	--
3,17-dione / 17-one	0.76	--	--	0.44	--	--

TABLE 13 - NGSeb 1%
(cont.)

Functional Group	5α			5β		
	Free	Acet	TMSi	Free	Acet	TMSi
17β-ol						
17β-ol / Androstane	0.53	0.52	0.25	0.82	0.82	0.51
3β,17β-diol / 3β-ol	0.65	0.22	0.28	0.64	--	-.06
3-one,17β-ol / 3-one	0.67	0.64	0.30	0.67	0.66	0.32
17α-ol						
3-one,17α-ol / 3-one	-	-	-	.64	.58	.18
3β-ol						
3β-ol / Androstane	0.55	0.56	0.28	0.51	0.53	0.52
3β,17β-diol / 17β-ol	0.67	0.26	0.32	0.33	-	-.05
3β-ol,17-one / 17-one	0.68	0.68	0.35	0.35	0.34	0.03
3α-ol						
3α,17β-diol / 17β-ol	0.61	0.21	0.18	0.36	0.02	0.01
3α-ol,17-one / 17-one	0.62	0.58	0.19	0.37	0.36	0.03
17-one						
17-one / Androstane	0.46	--	--	0.75	--	--
3β-ol,17-one / 3β-ol	0.58	0.58	0.53	0.59	0.56	0.27
3,17-dione / 3-one	0.61	--	--	0.63	--	--
3-one						
3-one / Androstane	0.55	--	--	0.54	--	--
3-one,17β-ol / 17β-ol	0.69	0.67	0.60	0.39	0.38	0.35
3,17-dione / 17-one	0.71	--	--	0.42	--	--

TABLE 14

Comparison of Calculated[a] and Observed Retention Time Values

Relative to Cholestane

Steroids	3% SE-30		3% XE-60		3% Hi-Eff 8B		3% NGSeb	
FREE	Calcd.	Obsd.	Calcd.	Obsd.	Calcd.	Obsd.	Calcd.	Obsd.
5α-A-3,17-dione	0.60	0.58	2.88	2.64	4.17	4.00	2.19	2.27
5β-A-3,17-dione	0.50	0.50	2.52	2.48	3.17	3.64	1.59	1.98
5α-A-3-one,17β-ol	0.63	0.59	2.58	2.58	4.68	4.61	2.57	2.61
5β-A-3-one,17β-ol	0.50	0.54	2.24	2.44	3.32	4.18	1.74	2.20
ACETATES								
5α-A-3β,17β-diol	1.00	0.98	2.75	2.21	3.39	3.18	2.19	2.20
5β-A-3β,17β-diol	0.81	0.86	2.24	1.90	-	2.42	-	1.66
TMSi Ether								
5α-A-3β,17β-diol	0.69	0.69	0.71	0.66	-	0.42	0.40	0.44
5β-A-3β,17β-diol	-	-	0.47	0.51	-	0.28	-	0.28

[a] Based on average log k values.

TABLE 15 - SE-30 3%[a]

Ratio of Relative Retention Times of Derivatives to Free Steroids

Steroids	Acetate	TMSi	TFA
5α-Androstane	-	-	-
5β-Androstane	-	-	-
5α-A-17β-ol	1.22	1.02	-
5β-A-17β-ol	1.27	1.19	-
5α-A-17-one	-	-	-
5β-A-17-one	-	-	-
5α-A-3β-ol	1.25	1.09	0.75
5β-A-3β-ol	1.22	1.09	0.75
5α-A-3-one	-	-	-
5β-A-3-one	-	-	-
5α-A-3α,17β-diol	1.65	1.09	0.52
5β-A-3α,17β-diol	1.69	1.09	0.63
5α-A-3β,17β-diol	1.68	1.17	0.62
5β-A-3β,17β-diol	1.75	1.14	-
5α-A-3α-ol,17-one	1.13	0.91	-
5β-A-3α-ol,17-one	1.20	0.98	0.60
5α-A-3β-ol,17-one	1.89	0.99	0.54
5β-A-3β-ol,17-one	-	-	-
5α-A-3-one,17β-ol	1.30	1.09	0.60
5β-A-3-one,17β-ol	1.28	1.09	0.61
5α-A-3-one,17α-ol	1.32	1.07	-
5β-A-3-one,17α-ol	1.14	0.90	0.62
5α-A-3,17-dione	-	-	-
5β-A-3,17-dione	-	-	-

TABLE 15 - XE-60 3%[b]
(Cont.)

Steroids	Acetate	TMSi
5α-Androstane	-	-
5β-Androstane	-	-
5α-A-17β-ol	1.18	0.63
5β-A-17β-ol	1.17	0.58
5α-A-17-one	-	-
5β-A-17-one	-	-
5α-A-3β-ol	1.08	0.59
5β-A-3β-ol	1.11	0.60
5α-A-3-one	-	-
5β-A-3-one	-	-
5α-A-3α,17β-diol	1.23	0.29
5β-A-3α,17β-diol	1.16	0.30
5α-A-3β,17β-diol	1.17	0.30
5β-A-3β,17β-diol	1.22	0.33
5α-A-3α-ol,17-one	1.07	0.44
5β-A-3α-ol,17-one	1.13	0.50
5α-A-3β-ol,17-one	1.16	0.55
5β-A-3β-ol,17-one	1.51	0.43
5α-A-3-one,17β-ol	1.09	0.51
5β-A-3-one,17β-ol	1.04	0.48
5α-A-3-one,17α-ol	1.14	0.57
5β-A-3-one,17α-ol	1.00	0.43
5α-A-3,17-dione	-	-
5β-A-3,17-dione	-	-

TABLE 15 - Hi-Eff 3%[c]
(Cont.)

Steroids	Acetate	TMSi	TFA
5α-Androstane	-	-	-
5β-Androstane	-	-	-
5α-A-17β-ol	0.82	0.31	0.23
5β-A-17β-ol	0.86	-	-
5α-A-17-one	-	-	-
5β-A-17-one	-	-	-
5α-A-3β-ol	0.95	0.32	0.27
5β-A-3β-ol	0.93	-	0.25
5α-A-3-one	-	-	-
5β-A-3-one	-	-	-
5α-A-3α,17β-diol	0.63	0.07	0.07
5β-A-3α,17β-diol	0.73	0.10	0.08
5α-A-3β,17β-diol	0.76	0.10	0.06
5β-A-3β,17β-diol	0.69	0.08	0.07
5α-A-3α-ol,17-one	0.70	0.20	0.21
5β-A-3α-ol,17-one	0.80	0.29	0.17
5α-A-3β-ol,17-one	0.87	0.30	0.17
5β-A-3β-ol,17-one	-	-	-
5α-A-3-one,17β-ol	0.84	0.28	0.27
5β-A-3-one,17β-ol	0.82	0.27	0.27
5α-A-3-one,17α-ol	0.76	0.25	-
5β-A-3-one,17α-ol	0.73	0.20	0.20
5α-A-3,17-dione	-	-	-
5β-A-3,17-dione	-	-	-

TABLE 15 - Hi-Eff 1%[d]
(Cont.)

Steroid	Acetate	TMSi	TFA
5α-Androstane	-	-	-
5β-Androstane	-	-	-
5α-A-17β-ol	0.88	0.39	0.23
5β-A-17β-ol	0.79	0.31	-
5α-A-17-one	-	-	-
5β-A-17-one	-	-	-
5α-A-3β-ol	0.89	0.43	0.27
5β-A-3β-ol	0.90	0.40	0.25
5α-A-3-one	-	-	-
5β-A-3-one	-	-	-
5α-A-3α,17β-diol	0.70	0.27	0.07
5β-A-3α,17β-diol	0.79	0.33	0.08
5α-A-3β,17β-diol	0.79	0.33	0.05
5β-A-3β,17β-diol	0.76	0.29	0.06
5α-A-3α-ol,17-one	0.84	0.27	0.21
5β-A-3α-ol,17-one	0.89	0.34	0.17
5α-A-3β-ol,17-one	0.91	0.35	0.17
5β-A-3β-ol,17-one	-	-	-
5α-A-3-one,17β-ol	0.85	0.32	0.27
5β-A-3-one,17β-ol	0.87	0.32	0.27
5α-A-3-one,17α-ol	0.92	0.35	-
5β-A-3-one,17α-ol	0.79	0.26	0.20
5α-A-3,17-dione	-	-	-
5β-A-3,17-dione	-	-	-

TABLE 15 - NGSeb 3%[e]
(Cont.)

Steroids	Acetate	TMSi	TFA
5α-Androstane	-	-	-
5β-Androstane	-	-	-
5α-A-17β-ol	0.94	0.43	0.34
5β-A-17β-ol	0.95	-	0.38
5α-A-17-one	-	-	-
5β-A-17-one	-	-	-
5α-A-3β-ol	1.00	0.45	0.38
5β-A-3β-ol	0.97	-	0.34
5α-A-3-one	-	-	-
5β-A-3-one	-	-	-
5α-A-3α,17β-diol	0.74	0.14	0.13
5β-A-3α,17β-diol	0.91	0.17	0.16
5α-A-3β,17β-diol	0.91	0.18	0.17
5β-A-3β,17β-diol	0.86	0.15	0.15
5α-A-3α-ol,17-one	0.88	0.32	0.37
5β-A-3α-ol,17-one	0.98	0.40	0.41
5α-A-3β-ol,17-one	0.95	0.43	0.45
5β-A-3β-ol,17-one	-	-	-
5α-A-3-one,17β-ol	0.91	0.41	0.45
5β-A-3-one,17β-ol	0.90	0.41	0.44
5α-A-3-one,17α-ol	0.95	0.42	-
5β-A-3-one,17α-ol	0.86	0.30	0.24
5α-A-3,17-dione	-	-	-
5β-A-3,17-dione	-	-	-

TABLE 15 - NGSeb 1%[f]

Steroids	Acetate	TMSi
5α-Androstane	-	-
5β-Androstane	-	-
5α-A-17β-ol	0.98	0.52
5β-A-17β-ol	0.10	0.49
5α-A-17-one	-	-
5β-A-17-one	-	-
5α-A-3β-ol	1.02	0.54
5β-A-3β-ol	1.05	-
5α-A-3-one	-	-
5β-A-3-one	-	-
5α-A-3α,17β-diol	0.39	0.19
5β-A-3α,17β-diol	0.46	0.22
5α-A-3β,17β-diol	0.38	0.23
5β-A-3β,17β-diol	-	0.21
5α-A-3α-ol,17-one	0.92	0.37
5β-A-3α-ol,17-one	0.99	0.46
5α-A-3β-ol,17-one	1.02	0.48
5β-A-3β-ol,17-one	0.97	0.48
5α-A-3-one,17β-ol	0.93	0.43
5β-A-3-one,17β-ol	0.97	0.44
5α-A-3-one,17α-ol	-	-
5β-A-3-one,17α-ol	0.89	0.35
5α-A-3,17-dione	-	-
5β-A-3,17-dione	-	-

Retention time of Cholestane: [a]12-14, [b]6-9, [c]6-8, [d]5-7, [e]9-11, [f]10 minutes.

TABLE -16

The Average Change in Retention Time Occurring in Derivative Formation

(r_{deriv}/ r_{free} x 100 = r',%)

Column	Acetate		TMSi		TFA	
	Mono	Di	Mono	Di	Mono	Di
SE-30 3%	123	169	102	115	69	59
XE-60 3%	110	120	53	30	--	--
Hi-Eff 8B 3%	83	70	27	9	23	7
NGSeb 3%	93	85	41	16	40	15

TABLE 17

Values of T at 260° Based on Measurements Obtained with 3% SE-30 and 3% XE-60 Columns

$$\left(T = \frac{t'_{XE\text{-}60} - t'_{SE\text{-}30}}{t'_{SE\text{-}30}} \right)$$

Functional Group	5α	5β
17β-ol	0.88	1.14
17-one	1.03	1.26
3β-ol	1.09	0.83
3-one	1.33	1.13
3α,17β-diol	2.09	3.08
3β,17β-diol	2.21	2.19
3α-ol,17-one	2.92	3.04
3β-ol,17-one	2.82	-
3-one,17β-ol	3.38	3.52
3-one,17α-ol	2.63	3.04
3,17-dione	3.55	3.98

TABLE 18

Comparison of Selective Retention of the Four Phases

for Ketone and Hydroxyl Groups

	Relative Retention Times			
Steroids	XE-60[a]	SE-30[b]	NGSeb[c]	Hi-Eff[d]
5α-Androstan-3β-ol	0.40	0.32	0.47	0.75
5β-Androstan-3β-ol	0.35	0.26	0.39	0.63
5α-Androstan-3-one	0.98	0.36	0.48	0.78
5β-Androstan-3-one	0.88	0.31	0.42	0.70

[a] Cholestane 9 minutes. 3% XE-60 on 80-100 mesh Diatoport S.

[b] Cholestane 13.6 minutes. 1% SE-30 on 80-90 mesh Anakrom ABS.

[c] Cholestane 10 minutes. 1% Neopentyl glycol sebacate on 80-90 mesh

[d] Cholestane 6.3 minutes. 1% Hi-Eff 8B on 80-100 mesh Diatoport S.

The $\triangle$ log r parameters for the oxidation of a hydroxyl group to a ketone are given in Table 19.

Separation factors of androstane isomers calculated according to the equation

$$r_{5\alpha} / r_{5\beta} = \text{separation factor}$$

are given in Table 20.

Discussion of Comparative Experiments

This study has confirmed the observations of Lipsky and Landowne (83) with respect to the properties of the neopentylglycol sebacate phase. In concentrations as low as one per cent (w/w) reproducible results were obtained with the neopentylglycol sebacate over a period of two to three months. Furthermore, after the initial curing process, this column could be used any time thereafter with remarkable reproducibility of initial retention data, a fact which may be indicative of the absence of any further loss of the phase during repeated use.

The Hi-Eff 8B phase, a polymer of dimethylcyclohexylsuccinate, exhibited about the same degree of polarity and thermal stability as did neopentylglycol sebacate. It is of interest that while there was no observable difference in the performance and characteristics of the one and three per cent neopentylglycol sebacate columns, some variation in the retention pattern between the one and three per cent Hi-Eff 8B

TABLE 19 - SE-30 3%

The Change in log r Contribution from Hydroxyl to Ketone Transitions

(Log $\triangle$r OH $\rightarrow$ one)

Functional Groups	5α	5β
17β-ol / 17-one	0	0.01
3β-ol / 3-one	0.02	0.04
3α-ol-17-one / 3,17-dione	0.05	0.03
3β-ol-17-one / 3,17-dione	0.02	-
3-one-17β-ol / 3,17-dione	0.01	0.03
3-one-17α-ol / 3,17-dione	0.01	0.02
3α,17β-diol / 3,17-dione	0.03	0.01
3α,17β-diol / 3α-ol,17-one	0.02	0.04
3α,17β-diol / 3-one,17β-ol	0.04	0.02
3β,17β-diol / 3,17-dione	0.01	0.01
3β,17β-diol / 3β-ol-17-one	0.02	-
3β,17β-diol / 3-one-17β-ol	0	0.04

TABLE 19 - XE-60 3%

(cont.)

Functional Groups	5α	5β
17β-ol / 17-one	0.50	0.20
3β-ol / 3-one	0.40	0.40
3α-ol-17-one / 3,17-dione	0.19	0.19
3β-ol-17-one / 3,17-dione	0.17	0.17
3-one-17β-ol / 3,17-dione	0.08	0.08
3-one-17α-ol / 3,17-dione	0.18	0.14
3α,17β-diol / 3,17-dione	0.27	0.24
3α,17β-diol / 3α-ol,17-one	0.08	0.06
3α,17β-diol / 3-one,17β-ol	0.19	0.17
3β,17β-diol / 3,17-dione	0.23	0.27
3β,17β-diol / 3β-ol-17-one	0.07	0.10
3β,17β-diol / 3-one-17β-ol	0.16	0.19

TABLE 19 - Hi-Eff 3%
(cont.)

Functional Groups	5α	5β
17β-ol / 17-one	0.06	0.11
3β-ol / 3-one	0.01	0.04
3α-ol-17-one / 3,17-dione	0.03	0.06
3β-ol-17-one / 3,17-dione	0	-
3-one-17β-ol / 3,17-dione	0.06	0.06
3-one-17α-ol / 3,17-dione	0.03	0.03
3α,17β-diol / 3,17-dione	0.01	0.02
3α,17β-diol / 3α-ol,17-one	0.02	0.03
3α,17β-diol / 3-one,17β-ol	0.07	0.04
3β,17β-diol / 3,17-dione	0.02	0.02
3β,17β-diol / 3β-ol-17-one	0.02	-
3β,17β-diol / 3-one-17β-ol	0.04	0.08

TABLE 19 - Hi-Eff 1%

(cont.)

Functional Groups	5α	5β
17β-ol / 17-one	0.07	0.06
3β-ol / 3-one	0.02	0.05
3α-ol-17-one / 3,17-dione	0.02	0.06
3β-ol-17-one / 3,17-dione	0.05	-
3-one-17β-ol / 3,17-dione	0.02	0.02
3-one-17α-ol / 3,17-dione	0.06	0.01
3α,17β-diol / 3,17-dione	0.05	0.02
3α,17β-diol / 3α-ol,17-one	0.05	0.04
3α,17β-diol / 3-one,17β-ol	0.07	0.04
3β,17β-diol / 3,17-dione	0	0.05
3β,17β-diol / 3β-ol-17-one	0.05	-
3β,17β-diol / 3-one-17β-ol	0.03	0.08

TABLE 19 - NGSeb 3%
(cont.)

Functional Groups	5α	5β
17β-ol / 17-one	0.08	0.07
3β-ol / 3-one	0.02	0.04
3α-ol-17-one / 3,17-dione	0.09	0.04
3β-ol-17-one / 3,17-dione	0.03	0.02
3-one-17β-ol / 3,17-dione	0.06	0.04
3-one-17α-ol / 3,17-dione	0.01	0.02
3α,17β-diol / 3,17-dione	0	0.02
3α,17β-diol / 3α-ol,17-one	0.09	0.06
3α,17β-diol / 3-one,17β-ol	0.06	0.02
3β,17β-diol / 3,17-dione	0.03	0.01
3β,17β-diol / 3β-ol-17-one	0.06	0.01
3β,17β-diol / 3-one-17β-ol	0.03	0.06

TABLE 19 - NGSeb 1%
(cont.)

Functional Groups	5α	5β
17β-ol / 17-one	0.07	0.07
3β-ol / 3-one	0	0.03
3α-ol-17-one / 3,17-dione	0.09	0.05
3β-ol-17-one / 3,17-dione	0.03	0.07
3-one-17β-ol / 3,17-dione	0.06	0.04
3-one-17α-ol / 3,17-dione	-	0.01
3α,17β-diol / 3,17-dione	0.03	0.01
3α,17β-diol / 3α-ol,17-one	0.06	0.06
3α,17β-diol / 3-one,17β-ol	0.08	0.03
3β,17β-diol / 3,17-dione	0.03	0.02
3β,17β-diol / 3β-ol-17-one	0.06	0.05
3β,17β-diol / 3-one-17β-ol	0.02	0.06

TABLE 20 - SE-30 3%

Isomeric Separation Factors
(5α / 5β)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane/5β-Androstane	1.04	-	-	-
5α-A-17β-ol/5β-A-17β-ol	0.80	0.76	-	-
5α-A-17-one/5β-A-17-one	0.76	-	-	-
5α-A-3β-ol/5β-A-3β-ol	1.09	1.11	-	1.08
5α-A-3-one/5β-A-3-one	1.03	-	-	-
5α-A-3α,17β-diol/5β-A-3α,17β-diol	1.04	1.02	0.95	0.87
5α-A-3β,17β-diol/5β-A-3β,17β-diol	1.19	1.14	1.22	-
5α-A-3α-ol,17-one/5β-A-3α-ol,17-one	1.10	1.03	1.02	-
5α-A-3β-ol,17-one/5β-A-3β-ol,17-one	-	-	-	-
5α-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.85	0.90	0.86	-
5β-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.77	0.87	0.84	0.85
5α-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	0.91	1.02	1.00	0.91
5β-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	-	-	-	-
5α-A-3-one,17β-ol/5β-A-3-one,17β-ol	1.08	1.10	1.08	1.06
5α-A-3-one,17α-ol/5β-A-3-one,17α-ol	1.06	1.23	1.26	-
5α-A-3,17-dione/5β-A-3,17-dione	-	-	-	-

TABLE 20 - XE-60 3%
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane/5β-Androstane	1.08	-	-	-
5α-A-17β-ol/5β-A-17β-ol	0.70	0.68	0.73	-
5α-A-17-one/5β-A-17-one	0.68	-	-	-
5α-A-3β-ol/5β-A-3β-ol	1.23	1.17	1.19	-
5α-A-3-one/5β-A-3-one	1.13	-	-	-
5α-A-3α,17β-diol/5β-A-3α,17β-diol	0.99	1.05	0.98	-
5α-A-3β,17β-diol/5β-A-3β,17β-diol	1.21	1.16	1.29	-
5α-A-3α-ol,17-one/5β-A-3α-ol,17-one	1.05	1.00	0.93	-
5α-A-3β-ol,17-one/5β-A-3β-ol,17-one	1.06	0.82	1.35	-
5α-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	1.00	0.90	0.79	-
5β-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.95	0.90	0.85	-
5α-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	1.04	1.03	1.05	-
5β-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	0.99	1.25	0.78	-
5α-A-3-one,17β-ol/5β-A-3-one,17β-ol	1.06	1.11	1.12	-
5α-A-3-one,17α-ol/5β-A-3-one,17α-ol	0.98	1.08	1.28	-
5α-A-3,17-dione/5β-A-3,17-dione	-	-	-	-

TABLE 20 - Hi-Eff 8B 3%
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane/5β-Androstane	1.06	-	-	-
5α-A-17β-ol/5β-A-17β-ol	0.48	0.46	-	-
5α-A-17-one/5β-A-17-one	0.53	-	-	-
5α-A-3β-ol/5β-A-3β-ol	1.23	1.25	-	1.33
5α-A-3-one/5β-A-3-one	1.09	-	-	-
5α-A-3α,17β-diol/5β-A-3α,17β-diol	1.02	0.88	0.76	0.90
5α-A-3β,17β-diol/5β-A-3β,17β-diol	1.20	1.33	1.50	1.03
5α-A-3α-ol,17-one/5β-A-3α-ol,17-one	1.04	0.92	0.72	1.31
5α-A-3β-ol,17-one/5β-A-3β-ol,17-one	-	-	-	-
5α-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.93	0.78	0.64	0.78
5β-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.90	0.85	0.89	0.60
5α-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	0.99	1.02	1.03	0.66
5β-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	-	-	-	-
5α-A-3-one,17β-ol/5β-A-3-one,17β-ol	1.10	1.13	1.12	1.09
5α-A-3-one,17α-ol/5β-A-3-one,17α-ol	1.10	1.14	1.35	-
5α-A-3,17-dione/5β-A-3,17-dione	1.10	-	-	-

TABLE 20 - Hi-Eff 8B 1%
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane/5β-Androstane	1.04	-	-	-
5α-A-17β-ol/5β-A-17β-ol	0.56	0.62	0.71	-
5α-A-17-one/5β-A-17-one	0.55	-	-	-
5α-A-3β-ol/5β-A-3β-ol	1.19	1.18	1.28	1.33
5α-A-3-one/5β-A-3-one	1.11	-	-	-
5α-A-3α,17β-diol/5β-A-3α,17β-diol	1.01	0.90	0.82	0.90
5α-A-3β,17β-diol/5β-A-3β,17β-diol	1.23	1.28	1.37	1.04
5α-A-3α-ol,17-one/5β-A-3α-ol,17-one	0.99	0.93	0.79	1.31
5α-A-3β-ol,17-one/5β-A-3β-ol,17-one	0.94	0.82	1.54	-
5α-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.87	0.83	0.68	0.78
5β-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.88	0.89	0.87	0.60
5α-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	0.98	1.01	1.00	-
5β-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	1.04	1.23	0.66	0.65
5α-A-3-one,17β-ol/5β-A-3-one,17β-ol	1.10	1.18	1.10	1.09
5α-A-3-one,17α-ol/5β-A-3-one,17α-ol	0.98	1.13	1.31	-
5α-A-3,17-dione/5β-A-3,17-dione	1.09	-	-	-

TABLE 20 - NGSeb 3%
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane/5β-Androstane	1.13	-	-	-
5α-A-17β-ol/5β-A-17β-ol	0.56	0.55	-	0.50
5α-A-17-one/5β-A-17-one	0.55	-	-	-
5α-A-3β-ol/5β-A-3β-ol	1.24	1.27	-	1.38
5α-A-3-one/5β-A-3-one	1.17	-	-	-
5α-A-3α,17β-diol/5β-A-3α,17β-diol	1.09	0.89	0.89	0.85
5α-A-3β,17β-diol/5β-A-3β,17β-diol	1.26	1.33	1.57	1.46
5α-A-3α-ol,17-one/5β-A-3α-ol,17-one	1.02	0.91	0.81	0.92
5α-A-3β-ol,17-one/5β-A-3β-ol,17-one	1.12	-	-	-
5α-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.88	0.81	0.64	0.80
5β-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.86	0.88	0.80	0.87
5α-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	1.00	1.01	1.00	1.12
5β-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	0.90	-	-	-
5α-A-3-one,17β-ol/5β-A-3-one,17β-ol	1.19	1.20	1.18	1.22
5α-A-3-one,17α-ol/5β-A-3-one,17α-ol	1.08	1.20	1.52	-
5α-A-3,17-dione/5β-A-3,17-dione	1.15	-	-	-

TABLE 20 - NGSeb 1%
(cont.)

Steroids	Free	Acetate	TMSi	TFA
5α-Androstane/5β-Androstane	1.13	-	-	-
5α-A-17β-ol/5β-A-17β-ol	0.58	0.56	0.61	-
5α-A-17-one/5β-A-17-one	0.57	-	-	-
5α-A-3β-ol/5β-A-3β-ol	1.23	1.20	0.65	-
5α-A-3-one/5β-A-3-one	1.14	-	-	-
5α-A-3α,17β-diol/5β-A-3α,17β-diol	1.03	0.87	0.90	-
5α-A-3β,17β-diol/5β-A-3β,17β-diol	1.26	-	1.43	-
5α-A-3α-ol,17-one/5β-A-3α-ol,17-one	1.03	0.96	0.82	-
5α-A-3β-ol,17-one/5β-A-3β-ol,17-one	1.21	1.27	1.19	-
5α-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.88	0.84	0.71	0.80
5β-A-3α-ol,17-one/A-5-ene-3β-ol,17-one	0.86	0.88	0.87	0.72
5α-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	1.00	1.06	1.02	-
5β-A-3β-ol,17-one/A-5-ene-3β-ol,17-one	0.82	0.83	0.88	0.76
5α-A-3-one,17β-ol/5β-A-3-one,17β-ol	1.15	1.10	1.10	-
5α-A-3-one,17α-ol/5β-A-3-one,17α-ol	-	-	-	-
5α-A-3,17-dione/5β-A-3,17-dione	1.12	-	-	-

columns was observed. One explanation for the change in the retention pattern of steroids on the one per cent Hi-Eff 8B column might be the presence of uncoated support surfaces. Either the viscosity of the polyester or possibly the porosity of the Diataport S support may result in incomplete covering of the solid surface by the liquid polymer.

XE-60, a recently developed silicone co-polymer consisting of equal amounts of dimethylsiloxane and cyanoethylmethylsiloxane, exhibited selective retention of compounds containing ketone substituents. Even though the experience of the author with this stationary phase has been most satisfactory, a word of caution seems necessary. Changes in the degree of selectivity which were observed after a few weeks of constant use at 260° might be indicative of loss of stationary phase or loss of a volatile component which was responsible for selective ketone retention. However, excellent stability of this phase at 350° was reported (87).

The least polar and the least selective phase studied was the methyl silicone gum SE-30. This elastomer was characterized by high thermal stability over a long period of time and exhibited retention which was directly proportional to the molecular weight of the solute. Wotiz (88), using an SE-30 column, has reported the separation of several major estrogen derivatives, which differ in the number of acetoxy groups. It has been reported that the particular non-selective property of the SE-30 elastomer with respect to various functional groups might make

this an ideal stationary phase in the determination of a parameter called steroid number (89). During the comparative study each liquid phase was studied individually and mixed phases were not employed. It was felt that with a mixed stationary phase the contribution of one group might be masked while that of others might dominate. The possibility of employing mixed phases to effect separation is, however, a very attractive one in gas chromatography and might be the key to successful applications. Some investigators have reported the use of mixed packing but their results appear inconclusive (90).

Concentration of the Stationary Phase and Mesh Size of Support

The second consideration, after the choice of suitable stationary phase for the column, was the concentration of this phase. The dependence of the height equivalent to a theoretical plate to the amount of stationary phase is given in the van Deemter equation (22).

$$\bar{H} = 2\lambda d_p + \frac{2\gamma D_{gas}}{u} + \frac{8k'}{\pi^2 (1+k')^2} \frac{d_f^2}{D_{liq}} u$$

Experiments designed to test the interrelationship of the various terms of the above equation have been reported.

Bohemen and Purnell have reported that the eddy diffusion term (or the multiple path effect), which was considered to be independent of the velocity of the carrier gas, may actually be inversely proportional to it (24). Improved column efficiencies were achieved by a decrease in mesh size and an increase in uniformity of support.

Similarly, increased column efficiency consequent to reduced ratio of liquid phase to solid support was observed (91) and led to the use of low load columns of one to five per cent instead of the ten to thirty per cent range employed during the early stages of applications of gas-liquid chromatography. Recently Hishta and his co-workers have reported the reduction of the time required for analysis by the simultaneous reduction of temperature and concentration of stationary phase on column support (92).

A word of caution is necessary on low-load columns. Incomplete and non-uniform distribution within the support as a result of insufficient amount of liquid phase may lead to exposed sites on the column support. The result would be a system of not two but three components. This point was recently discussed by Keller and Stewart (93). According to these authors low load columns are usually of such a composition that neither the two-phase system of liquid partition nor that of a solid adsorption predominates and the description and treatment of these systems as two phase systems is incorrect.

Thus, while liquid-liquid distribution certainly dominates most successful solvent systems designed as partition systems, complications due to adsorption and other factors are quite probable and should not be minimized. Martin (94) has suggested that the low R_F values of basic amino acids in chromatograms based on starch or cellulose, which were smaller than the R_F values expected on the basis of partition

coefficients in the pure solvents of a given system, might be due to ion-exchange with carboxyl groups or to association with the partially dissolved polysaccharide molecules of the stationary phase.

While evidence for the thermal stability of steroids, which allows their vapor phase separations is abundant (95-97) occasional instances of alteration during gas chromatography of the more sensitive type of molecules such as the corticosteroids have been reported (98). The reason for this decomposition is not clear. Recently Wotiz (99) has reported incomplete coating of the column as a possible cause of the decomposition of steroid molecules. Wotiz studied the behavior of several types of steroids on column supports coated with stationary phases of various concentrations. Decomposition was observed on very low-load columns (0.25 - 0.50 w/w per cent) even for very stable steroids. A possible explanation suggested by Wotiz is that during gas chromatography the solute molecule which was activated in the vapor phase may be deactivated in the liquid phase by dissipating its energy of activation to the solvent molecules. If there is insufficient amount of solvent to absorb the activated molecule, thermal degradation might occur.

The interrelationships of the terms in the van Deemter equation and the effect of each term of the equation on $\bar{H}$ may be summarized as follows:

The smaller and more uniform the particle size of the solid support, the smaller is $\bar{H}$. The contribution of mesh size is negligible at low flow rates but becomes a major contributing factor to $\bar{H}$ at high rates of flow.

The smaller the concentration of the liquid coating on the column, the smaller is $\bar{H}$. When the range of flow is wide, a larger concentration of the liquid coating results in increased column efficiency. When the range of flow is narrow, better performance is achieved with low-load columns.

The smaller the quantity of the sample, the higher is the number of theoretical plates and the smaller is $\bar{H}$.

A clarification of the last point is necessary. The size of the sample is directly dependent on the concentration of the liquid phase. Heavily coated columns can tolerate larger amounts samples than can low-load columns. Therefore, a discussion of the influence of sample size is meaningful only as expressed simultaneously with the percentage of stationary phase.

The theoretical dependence of $\bar{H}$ upon experimental parameters in terms of the extended van Deemter equation and the experimental evaluation of rate equation constants are discussed in a comprehensive manner by Purnell (25).

On the basis of preliminary results obtained, liquid concentrations of three per cent and mesh range of 40-50 were chosen for the comparative study. These conditions could be altered in either direction if necessary. For the comparative study on structure of steroids and their chromatographic behavior the above conditions were found to be satisfactory for all columns. These conditions allowed a sufficient amount of phase for interactions in a partition system and a wide range for the variation of flow.

On the XE-60 columns the peaks were sharp and symmetrical. On the remaining three columns, free steroids and their acetylated derivatives tended to give wide peaks, especially for the dioxygenated compounds, but the trimethylsilyl and trifluoroacetate derivatives were eluted rapidly with sharp peaks.

The separation of the 17-ketosteroids and of the two pregnane isomers could be effected only on the XE-60 column and not on the other columns in spite of efforts to effect separations by variations of temperature and flow rate. Columns with one per cent liquid phase on finer mesh supports were constructed for the SE-30, neopentylglycol sebacate and Hi-Eff 8B phases.

As predicted by the van Deemter equation, the combined effect of the finer and more uniform particle size of the support and the reduced concentration of the stationary phase was to increase column resolution. It must be emphasized that column efficiency is not the sole factor of importance in gas-liquid chromatography. Some excellent separations can be achieved on columns not operating at maximum efficiency. Selective retention of the liquid plays a role here. Furthermore, column efficiency is not an absolute quantity but varies for different compounds. The van Deemter equation makes available to the investigator a tool for improving and revising column conditions and efficiency when desired.

Separation of Urinary 17-Ketosteroids (100)

Known Mixtures

A known mixture of the trimethylsilyl ethers of the principal 17-ketosteroids and two pregnane isomers were chromatographed in the usual manner on four columns.

A column of 1% SE-30 coated on 80-90 mesh Anakrom ABS in a 6-foot 1/8" (o.d.) stainless steel tube was prepared. The chromatograph equipped with the flame ionization detector was employed. Column conditions: T = 251°, P = 14 psi, Retention time for cholestane = 13.6 minutes.

Other columns used in the separation study were 1% Hi-Eff 8B, 1% NGSeb and 3% XE-60 under the same conditions as described above. Results are presented in Table 21. The five major urinary 17-ketosteroids were resolved on the XE-60, Hi-Eff 8B and NGSeb columns. Only partial separations were obtained on the SE-30 column.

Chromatograms showing the separation of the urinary ketosteroids and the progesterone metabolites are shown in Figures 9-11. The separation of the two pregnane isomers simultaneously with the 17-ketosteroids was achieved only on the Hi-Eff 8B and XE-60 columns.

TABLE 21

Separation of Principal 17-Ketosteroids and Progesterone Metabolites as Trimethylsilyl Ethers

Steroids	XE-60[a]	SE-30[b]	NGSeb[c]	Hi-Eff[d]
3α-Hydroxy-5α-androstan-17-one	1.02	0.47	0.60	0.78
3α-Hydroxy-5β-androstan-17-one	1.18	0.50	0.73	0.98
3β-Hydroxyandrost-5-en-17-one	1.36	0.56	0.84	1.14
3α-Hydroxy-5β-androstane-11,17-dione	2.40	-	1.36	2.03
3α-11β-Dihydroxy-5α-androstan-17-one	2.57	-	1.87	2.59
5β-Pregnane-3α,20α-diol	0.78	-	0.70	0.68
3α-Hydroxy-5β-pregnan-20-one	1.44	-	1.08	1.38

[a] Cholestane 9 minutes. 3% XE-60 on 80-100 Diatoport S.

[b] Cholestane 13.6 minutes. 1% SE-30 on 80-90 mesh Anakrom ABS.

[c] Cholestane 10 minutes. 1% Neopentyl glycol sebacate on 80-90 mesh.

[d] Cholestane 6.3 minutes. 1% Hi-Eff 8B on 80-100 mesh Diatoport S.

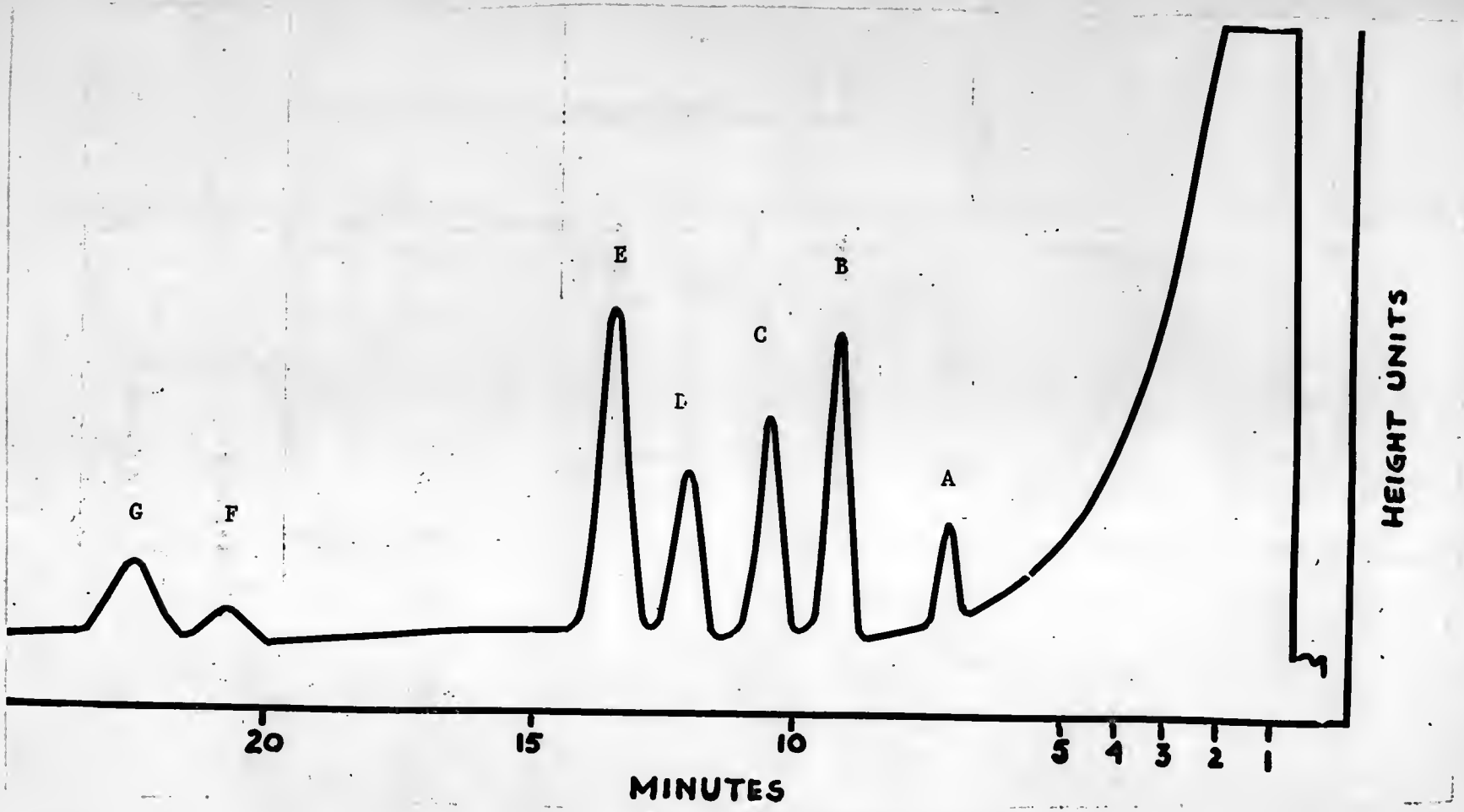

FIGURE 9

Gas-liquid chromatogram of a mixture of known steroid trimethylsilyl ethers on 3% XE-60 column. Conditions as described in text. (A) 5β-Pregnane-3α,20α-diol, (B) 3α-hydroxy-5α-androstan-17-one, (C) 3α-hydroxy-5β-androstan-17-one, (D) 3β-hydroxyandrost-5-en-17-one, (E) 3α-hydroxy-5β-pregnan-20-one, (F) 3α-hydroxy-5B-androstane-11,17-dione, (C) 3α,11β-dihydroxy-5α-androstan-17-one.

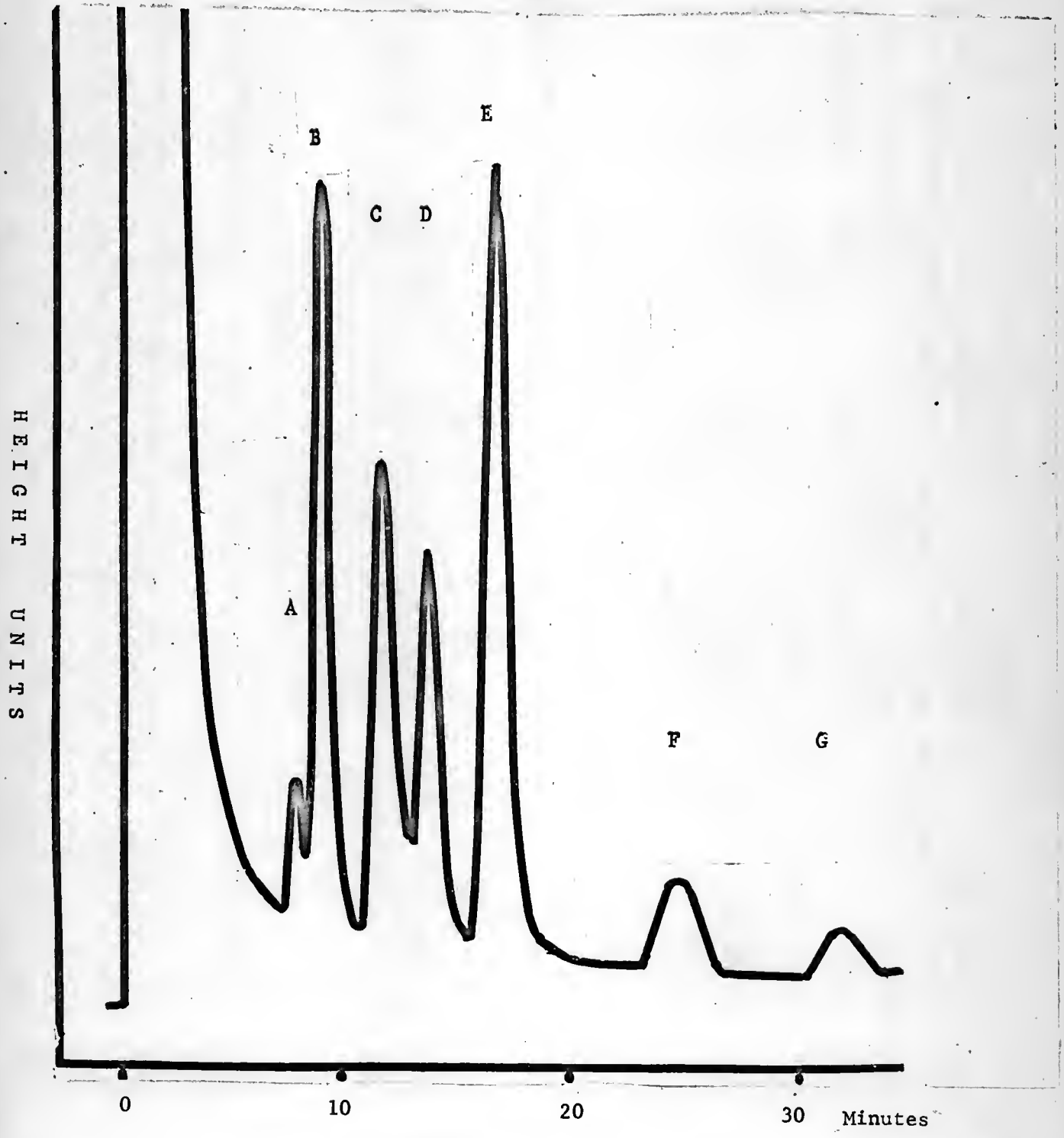

FIGURE 10

Gas-liquid chromatogram of a mixture of known steroid trimethylsilyl ethers on 1% Hi-Eff 8B column. Conditions as described in text. (A) 5β-Pregnane-3α,20α-diol. (B) 3α-hydroxy-5α-androstan-17-one, (C) 3α-hydroxy-5β-androstan-17-one, (D) 3β-hydroxyandrost-5-en-17-one, (E) 3α-hydroxy-5β-pregnan-20-one, (F) 3α-hydroxy-5β-androstane-11,17-dione, (G) 3α,11β-dihydroxy-5α-androstan-17-one.

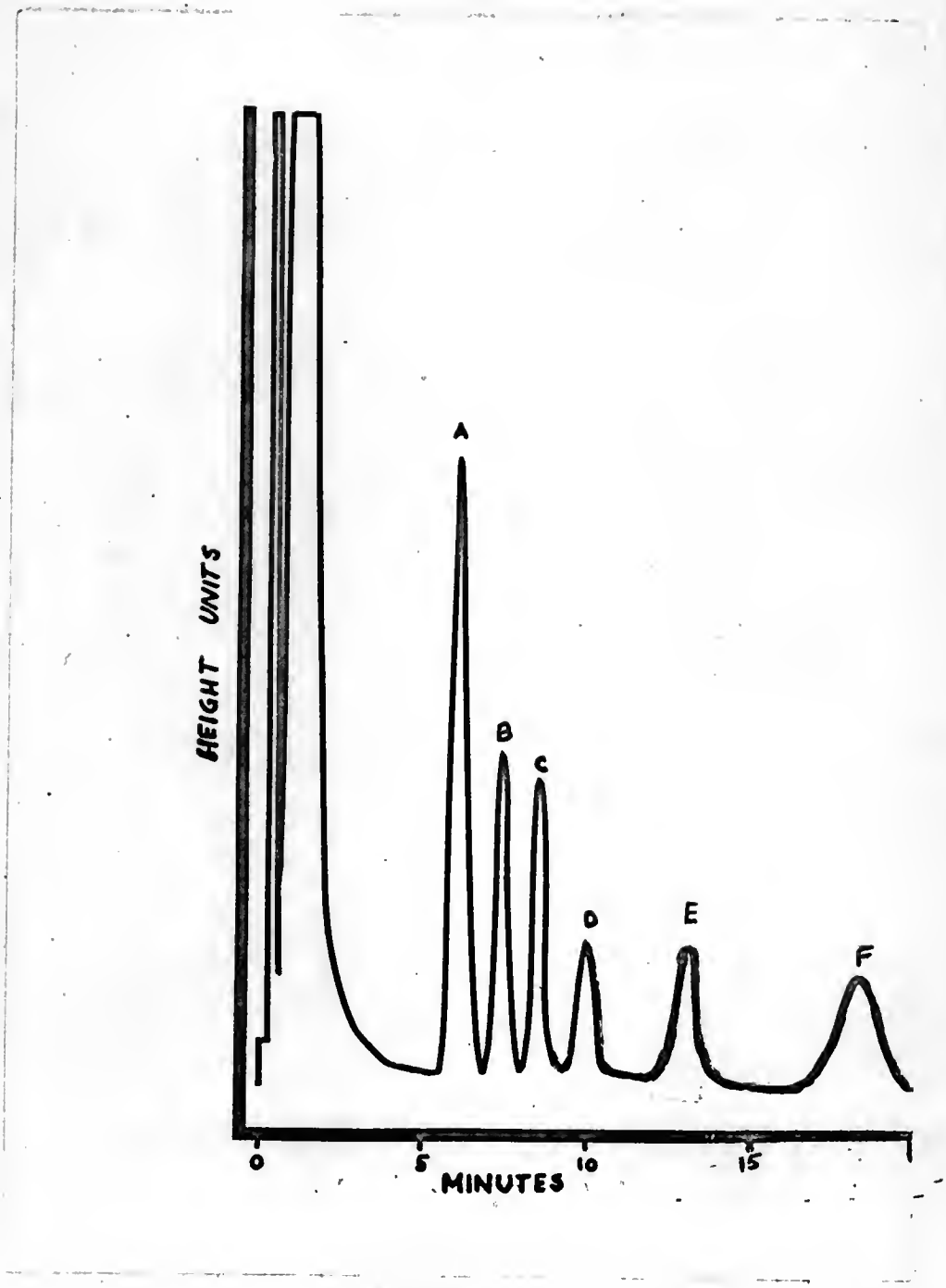

FIGURE 11

Gas-liquid chromatogram of a mixture of known steroid trimethylsilyl ethers on 1% NGSeb column. Conditions as described in text. (A) 3α-Hydroxy-5α-androstan-17-one, (B) 3α-hydroxy-5β-androstan-17-one, (C) 3β-hydroxyandrost-5-en-17-one, (D) 3α-hydroxy-5β-pregnan-20-one, (E) 3α-hydroxy-5β-androstane-11,17-dione, (F) 3α,11β-dihydroxy-5α-androstan-17-one.

Preparation of Urine Sample

A portion (24 ml) of a 24 hour specimen was treated with 2000 units/ml of helix pomatia extract (Glusulase, Endo Laboratories) at pH 4.8 and 37° for 24 hours followed by extraction with dichloromethane. The extract was washed with 0.1 N NaOH and water and the extract was carefully evaporated *in vacuo* to complete dryness. The residue was then treated with hexamethyldisilazane and trimethylchlorosilane as described under Materials and Methods. The chromatogram of a 1/2040 aliquot of the total urine is shown in Figure 12.

Discussion of the Separation of Urinary 17-Ketosteroids

All measurements were made on the trimethylsilyl derivatives. The five major urinary 17-ketosteroids and the two pregnane metabolites were separated on the Hi-Eff 8B column when the column conditions were adjusted to give maximum resolution for the 17-ketosteroids exclusively. The importance of low-load columns in achieving higher column resolution was thus evident. On the one per cent neopentylglycol sebacate column, separations could be accomplished for the five principal urinary ketosteroids, however, 3α-hydroxy-5β-androstan-17-one could not be separated from 5β-pregnane-3α,20α-diol. All seven steroids were resolved on the three per cent XE-60 column. Only partially resolved peaks could be effected on the one per cent SE-30 column. This result was not surprising since the non-selectivity properties of the SE-30 polymer

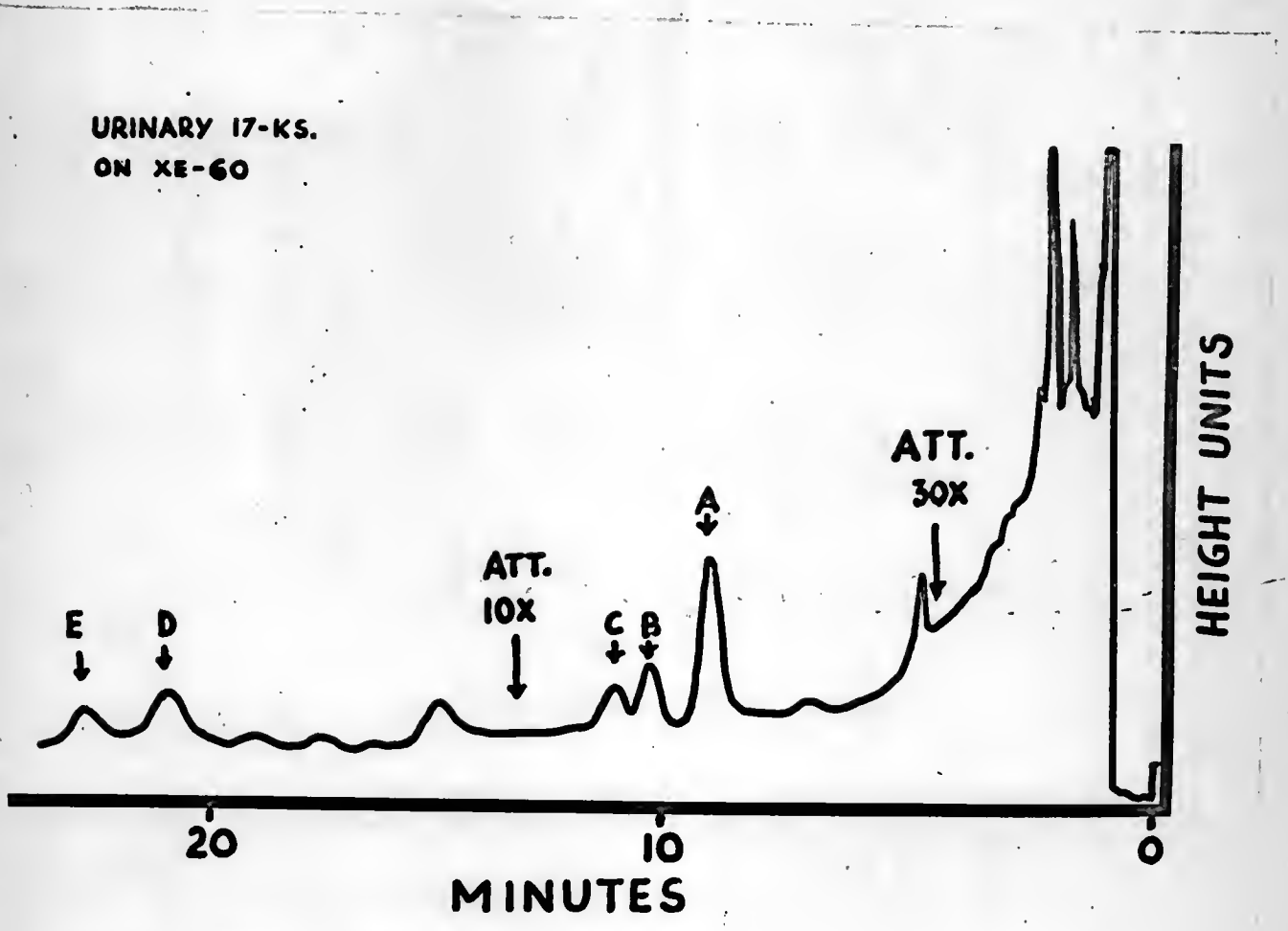

FIGURE 12

Gas-liquid chromatogram of urinary components as trimethylsilyl ethers on 3% XE-60 column. Conditions as described in text. (A) 3α-hydroxy-5α-androstan-17-one, (B) 3α-hydroxy-5β-androstan-17-one, (C) 3β-hydroxy-androst-5-en-17-one, (D) 3α-hydroxy-5β-androstane-11,17-dione, (E) 3α-11β-dihydroxy-5α-androstan-17-one.

were evident in the preliminary experiments. The purpose for including the SE-30 phase in the present investigation was primarily as a reference for comparison of the more selective phases.

Study of the Dependence of Relative Retention Time on Temperature and on the Nature of the Reference Standard

In order to assess the variation of relative retention time with changes in temperature the retention times of three androstane trimethylsilyl ethers and of cholestane were determined on the one per cent SE-30 column (described above) at four different temperatures. Relative retention times were then calculated first as related to cholestane and then as related to the trimethylsilyl ether of 3α-hydroxy-5α-androstan-17-one. The results are given in Table 22.

Discussion of Factors Affecting Retention Time Values

Temperature is a major factor which affects the elution time of a solute in a gas-liquid chromatographic column. The rate of flow is also a contributing factor to the retention time values. However, the rate of flow varies inversely with temperature, hence its effect is not always obvious.

While changes in absolute retention times with changes in temperature are normally expected, the variation of relative retention values with temperature poses a problem to the investigator. The changes in relative retention time over a twenty degree temperature range were recently reported (89) and the recommendation was made

TABLE 22

The Effect of the Reference Standard on the Variation of Relative Retention Time with Temperature on 1% SE-30 Column

Steroids	Temp., °C											
	251			240			230			221		
	a	b	c	a	b	c	a	b	c	a	b	c
(1) 5α-A-3α-ol,17-one	6.3	0.46	1.00	8.8	0.43	1.00	12.3	0.40	1.00	15.5	0.38	1.00
(2) 5β-A-3α-ol,17-one	6.8	0.50	1.08	9.4	0.46	1.07	13.0	0.42	1.06	16.5	0.40	1.07
(3) A-5-en-3β-ol,17-one	7.6	0.56	1.21	10.8	0.53	1.23	15.2	0.50	1.24	19.3	0.47	1.24
(4) Cholestane	13.6	-	-	20.4	-	-	30.6	-	-	41.0	-	-

a - Observed absolute retention time in minutes. b.- Retention relative to cholestane. c - Retention relative to (1).

that all measurements on all columns should be made at the same temperature. While such a precautionary measure might be desirable in comparative work, it may not always be feasible. For solutes of low mobility it is more advantageous to employ higher temperatures than higher flow rates, because retention time varies exponentially with temperature and linearly with the velocity of the carrier gas. Furthermore, high-load columns require higher temperatures than low-load columns.

An alternative to the constant temperature conditions as suggested by Vandenheuvel and Horning (89) is the use of a reference standard other than cholestane. If a standard structurally similar to the group of compounds under investigation is employed, then, under the same conditions, variations with temperature in the retention time of such a standard would be expected to be in approximately the same ratio as the variations in the retention time of the samples. The experiment designed to test this assumption supported the above argument (Table 22). When cholestane is used as the reference standard, the mean variation of the relative retention time over the 30° range is 3.5 per cent. When one of the androstane isomers is employed as the reference standard, the mean variation is 0.5 per cent.

The dependency of retention times on the quantity and volume of the sample was also reported by the same authors (89). Small

volumes and concentrations of samples preferrably in the same solvent were suggested. In the course of the present investigation 0.2 to 1 µl aliquots of 2 µg/µl concentrated solutions were chromatographed with good reproducibility of retention times. However, in the isolated instances where larger volumes were used, reproducibility of the data was not compromised. Large quantities of samples which adversely affect column efficiency should be avoided, expecially in low-load columns. Variation in retention times was not observed when different solvents were used. Nevertheless, it is desirable to employ the same solvent especially in comparative studies provided all of the solutes are soluble in one solvent.

DISCUSSION

Relationship of Chromatographic Behavior to Chemical Structure

Retention Patterns

Certain deductions concerning the polarities of the four stationary phases may be drawn from the relative retention times of the free steroids and the three derivatives (Table 11). The polarities of these phases are approximated as Hi-Eff 8B ≅ NGSeb > XE-60 > SE-30. The general pattern of relative retention time values is: free steroid > acetate > trimethylsilyl > trifluoroacetate for the two polyester columns; acetate > free steroid > trimethylsilyl for the XE-60 polymer; acetate > trimethylsilyl ≅ free steroid > trifluoroacetate for the non-polar SE-30 elastomer.

Derivatives have been employed since the early days of chromatography (101-106). The use of derivatives in steroid analysis was advocated by Wotiz (86,105) and VandenHeuvel et al.,(73,75,106). There are several advantages to the use of derivatives in preference to the free steroids. Derivatives are more stable to heat. Polarity can be varied by the formation of a derivative. Molecular

weight differences may result in better separations where differences in the number of substituent groups exist. Greater sensitivity may be obtained with derivatives when β-ionization detectors are employed (79).

The retention time of a solute in the chromatographic column is the sum of its residence time in each phase of the column and is dependent on the degree of interaction of the solute and the solvent. Hence, a change in the polarity of a molecule consequent to derivative. formation is reflected in its retention time. A substituent such as the acetyl group will decrease the polarity due to the hydroxyl group and contribute to an increased retention time value of the steroid acetate on non-polar columns and to a decreased value on polar columns. The results obtained in the present investigation were in agreement with the qualitative predictions.

In the case of trimethylsilyl ethers the great reduction in retention times on polar columns is due to the fact that the trimethylsilyl group behaves like a non-polar functional group. The group is sufficiently bulky to affect molecular weight and shape, but does not show selective functional-group retention effects. The high volatility of these ethers is indicated by the lower than expected retention times on the non-polar columns and by the unexpectedly low retention times on the polar columns.

The changes brought about in the steroid molecule by the substitution of the trifluoroacetyl group are ambiguous. In general, trifluoroacetyl derivatives have been found to be less satisfactory than the trimethylsilyl derivatives (107). The ease of decomposition of the trifluoroacetyl derivatives on chromatographic columns introduce some unreliability in their data. Research was not pursued on these derivatives.

One further observation emerges from the results summarized in Table 11. Changes in retention time values of the steroids consequent to derivative formation notwithstanding, the derivatives follow the retention pattern of the isomeric free steroids in all but a few instances. However, the retention time values of the isomeric derivatives do not always retain the same ratio to each other as do the corresponding free compounds. A similar observation was made by Lipsky and Landowne (83), who reported that the observed decreases in retention times of steroid acetates on polar columns were not the same in all cases. The reversal of the order of elution upon derivative formation was seen in the case of the isomeric 3α,17β-diol and 3α-ol,17-one pairs with almost all of the columns studied. However, no explanation was evident for this anomaly.

Effect of Structure on Retention Data

On all columns studied the retention time values of the 5α-androstane nucleus is consistently greater than that of the 5β-isomer as seen in Table 12. Although this difference in the values of the 5α- and the 5β-androstane nuclei is small, it is nevertheless constant and reproducible on all columns and is reflected in the retention time values of the oxygenated nuclei as well. A basic structural difference or a unique chemical property must account for the greater mobility of the 5β-androstane molecule. A possible explanation may lie in the planarity of the 5α-androstane nucleus. The wide area of the planar molecule enhances the interactions of the steroid with the liquid phase of the column, hence decreases the mobility of the 5α-compound.

The pattern established by the parent nuclei is followed by all the C-3 oxygen and C-3,17 dioxygen substituted isomers. The length of time by which the substituted 5β-steroids precede the 5α-isomers varies from column to column and the variation is not in the same ratio as those of the parent nuclei.

The isomeric pairs of androstan-17β-ol and androstan-17-one not only do not follow the pattern of their respective nuclei but reverse it markedly. Thus 17-oxygenated 5β-androstanes exhibit retention values nearly double those of the 5α-isomers. This reversal is most pronounced in the case of the two polar columns and less

pronounced for the XE-60 and SE-30 columns. It is evident that the presence of an oxygen function in the C-17 of the steroid nucleus strongly influences the elution ratio of the 5α/5β isomers and that this influence is dominant regardless of the nature of the liquid solvent. To our knowledge this is the first observation of the anomalous effect of the 17-oxygen function in androstanes or in any other class of steroids. The consistent observation of the effect of this group in all the solvents under study is in agreement with the general observation in other types of chromatographic studies. It has been reported (3) that changes in the R_F values brought about by the secondary hydroxyl groups in different positions on the steroid nucleus were usually the same for a variety of solvent systems.

The two isomeric pairs of 3,17-dioxygenated androstanes maintain the characteristic elution order of the hydrocarbon nuclei, a fact indicating an opposing effect of the oxygen function on carbon atom 3. However, a marked difference between the effect of the 3α- and 3β-hydroxyl function is evident from the results in Table 12. In all cases studied, the presence of the 3β-hydroxyl group appears to enhance the normal difference between the 5α- and 5β-isomers while the presence of the 3α-hydroxy group appears to counteract the influence of the 17-oxygen only to the point where the isomeric 3α,-17β-diol and 3α-ol,17-one androstanes exhibit nearly identical retention times. These observations were made with each column, including the SE-30 column in which case the influence of the 17-oxygen

function was found to be the least evident. Thus, the oxidation of the C-3 hydroxy function to the corresponding ketone brings about a significant change in retention time values only when compared to the 3α,17β-diol compound. The contribution of the 3β-hydroxyl group in increasing the retention value of the 5α-androstane isomers can also be deduced from the comparative data given in a recent paper for the purpose of illustrating certain other factors (107).

The influence of the 3β-hydroxyl group may be considered in terms of the conformations of the steroid molecules, that is, in terms of the orientation of the C-3 hydroxyl group within the 5α- and the 5β-series. A further correlation between chromatographic behavior, structure and conformation then emerges. In the 5β-isomers the 3β-hydroxyl isomers (axial conformation) exhibit greater mobility than the corresponding 3α-hydroxyl compounds (equatorial conformation). In the 5α-series, on the other hand, this pattern is reversed and compounds with the hydroxyl group in the 3α-orientation (axial conformation) exhibit greater mobility than their 3β-isomers (equatorial conformation). It is significant that in both series the orientation which is cis between the C-3 and C-5 results in greater mobility. It is possible that intramolecular interaction may exist between the C-5 hydrogen and the C-3 hydroxyl group. These observations are in agreement with those of Savard on the paper partition chromatography of C_{19} and C_{21} steroids (65) and with the general concept defined by Barton (31,66).

Theoretical Aspects of Partition Chromatography: Relationship of Structure to Chromatographic Mobility

Martin (94) has developed a theory of partition coefficients based on the assumption that the chemical potential of a substance is an additive function of the constituent parts of its molecule. If μ_A is the chemical potential of the substance A and $\Delta \mu_A$ is equal to the free energy required to transport one mole of A from one phase to another, then as a first approximation $\Delta \mu_A$ may be regarded as being equal to the sum of the potential differences ($\Delta \mu_a, \Delta \mu_b$) of the various groups which make up molecule A.

$$\Delta \mu_A = \Delta \mu_a + \Delta \mu_b + \Delta \mu_c$$

To a first approximation, the free energy required to transport a given group X from one solvent to another is independent of the rest of the molecule. The addition of a group X to a molecule will change the partition coefficient, and hence the retention time value, of a substance by a definite amount in a particular solvent system. This change will be dependent on the nature of group X and independent of the structural features of the rest of the molecule (94). Introduction of more than one group into a molecule will cause a change in the partition coefficient directly proportional to the number of groups of X.

Martin, in pointing out the limitations of the application of his theory, suggested that stereochemical factors might be a major cause of deviation from theory in the relationship of structure to partition coefficient. Owing to steric factors, the solvent molecules may be unable to approach the solute molecules and the energy of association between the solute and the solvent will be lower than expected from the considerations of the sum of various groups of the solute molecule. Bate-Smith and Westall (108) have reported excellent agreement with the theory in their study of a wide range of polyphenolic compounds with substituents where little or no steric hinderance was involved.

It has been observed that deviations from theory begin to appear when the solute contains fused ring systems. Since most steroid molecules contain at least four fused alicyclic rings, it is not surprising that in this class of compounds steric factors should be more pronounced and that more of the substituent groups should show deviations from theory. However, the steric limitations in the steroid molecule might introduce an advantage to the applicability of the theory to this group of compounds. The rigidly joined ring system of the steroid nucleus has little possibility of conformational inversion. Consequently the conformations of any one steroid are limited in number. Furthermore, substituents on the large extended steroid molecule might be expected to be sufficiently far apart to exert little or no influence on each other. This is an ideal

requirement for any application of the theory. (Note, however, the dependence of the influence of the 17-oxygen function on substitution at C-3.) Bush has made the practical suggestion that in order to make the theory workable over a wide range of steroids, the word "group" be defined to mean not only the chemical nature of the group, as suggested by Martin, but its position and orientation on the nucleus as well (109).

Martin's concept of the additive contribution to chromatographic mobility of the components of a molecule was recently applied to the behavior of steroid molecules during gas-liquid chromatography by Clayton (110-112) and Knights and Thomas (113). Clayton expressed the mobility of a substituted molecule in terms of the mobilites attributable to the components of that molecule.

$$r = r_n \times k_a \times k_b \ \ldots\ldots$$

where r_n is the retention time of the unsubstituted nucleus, and k_a, k_b etc. are group retention time factors of non-interacting groups at positions a, b on the nucleus. The contribution of the relatively non-polar methoxy group at C-3 was found to be constant while that of the polar 5α-hydroxyl group was not. Clayton suggested that the observed variation in the contribution of the 5α-hydroxyl group may be in part due to steric hinderance which restricts the interaction of the polar liquid phase with a double bond in the molecule.

Knights and Thomas (113) have used the above equation in its logarithmic form

$$\log r = \log r_n + \log k_a + \log k_b \ldots\ldots$$

These investigators have demonstrated that the contribution to retention time of the C-10 methyl group for several steroids was constant. Other observations of these authors include the constancy in the Δ log r contributions for C-3 equatorial hydroxyl to C-3 ketone transitions on a stationary phase selective for ketones. The vicinal group effects observed in the variation in the log r parameters of 11β-hydroxyl and 11-ketone groups were in keeping with the theory. A negative contribution of the C-16 methyl group was attributed to the change in the contribution of the 20-ketone in the 16α- and 16β-methyl pregn-4-ene-3,20-dione, as a result of steric hinderance. It must be emphasized, however, that not all discrepancies with respect to theory are explicable in terms of steric factor. The data of Knights and Thomas are too limited to permit such a generalization.

Calculated group retention factors (Table 13) obtained on the two polar phases exhibit similarities in the over-all pattern as well as in the individual values. Similarly, the values obtained on the two non-polar phases are alike, though some of the extremely low and high values obtained on the SE-30 column are moderated on the XE-60 column.

The magnitude of log k_a is determined by the extent of the interaction of group (a) with the liquid phase. Since functional group effects are accentuated on polar columns and minimized on non-polar ones, log k values of functional groups should be larger for polar solvents than for non-polar phases. The results suggest a greater polarity of the Hi-Eff 8B polyester as compared to the neopentylglycol sebacate phase.

A comparison of the group retention time factors of the acetyl versus the trimethylsilyl groups show better agreement among the log k_{acet} values, which is in agreement with other observations made on the acetate group(Table 16).

Retention time values were calculated using the average log k values obtained from Table 13 and compared with the observed retention time values (Table 14). In general, the agreement between the observed and the calculated figures is quite good. It is interesting that deviations occur when the influence of the C-17 oxygen function is most pronounced, that is, on the two polar columns for the 5β-androstanes.

The changes in retention time upon derivative formation are expressed as the ratio of the relative retention times of the derivatives to those of the free steroids (Table 15). Interactions of the non-polar acetate group with the non-polar SE-30 and to a lesser extent with the XE-60 phases are indicated in the increased retention

times of the steroid acetates on these columns. The decreased interactions of the acetates with the polar phases are reflected in the faster elution time of the steroid acetates as compared to the free steroid on the Hi-Eff 8B and NGSeb columns.

The average change brought about in the retention of the parent compound upon derivative formation is given in Table 16. It is evident that of the three derivatives, the contribution to retention time values is additive only in the case of the acetate group. The absence of a direct relationship between contribution to retention time and number of substituent groups in the case of trimethylsilyl ethers and the trifluoroacetates is indicative of the presence of factors such as volatility and steric hinderance. The use of the acetyl group in preference to the trimethylsilyl or trifluoroacetate derivatives for structural studies is suggested by these results.

VandenHeuvel and others (89,90) have suggested two new terms, steroid number (SN) and T, as parameters for the elucidation of the structural features of a steroid molecule. Both of these terms are directly derived from the work of Woodford and van Gent (114) in the gas-liquid chromatography of fatty acids. Steroid number is analogous to carbon number of fatty acids, which is related to retention time r by

$$\log r = k \times \text{no of C in fatty acid molecule}$$

The manner of determining the carbon number is to construct a reference graph according to the above equation based on the carbon number of known fatty acids. The carbon number of an unknown acid can then be read off at a point corresponding to the retention time of the acid. The concept of carbon number may be useful because it is independent of slight changes in temperature and eliminates the need for an arbitrary reference standard to which retention times must be related.

Steroid number is expressed as a summation of terms dependent on the nature of the carbon skeleton and the functional groups of the steroid molecule.

$$SN = S + F_a + F_b \ldots\ldots$$

where SN is the steroid number, S is the carbon content of the steroid skeleton and F_a, F_b are values characteristic of the functional groups of the steroids.

In the opinion of this investigator there are at least three drawbacks to the use of SN term for the elucidation of structure.

1. Steroid numbers are determined from relative retention values, so that the advantage cited by Woodford and van Gent in eliminating the reference standard no longer exists. Furthermore the temperature dependency of the relative retention time value will be present in the steroid number term.

2. Woodford and van Gent employed a calibration curve based on five fatty acids of known chain length. VandenHeuvel and Horning draw their standard curve between only two points. The reliability of any information derived from such a calibration graph is questionable.

3. The steroid number term neither reflects the actual carbon content of the steroid molecule nor indicates substituent groups on the nucleus with any accuracy. For example, the value of SN is 29.4 for cholestanol, 29.2 for cholestenyl methyl ether and 28.8 for cholestanyl trifluoroacetate. The term is therefore misleading.

The second parameter derived from the early work of Woodford and van Gent is called the T term which is a function of the selectivity of the stationary phase. This term is defined as

$$T = \frac{t'_s - t'_n}{t'_n}$$

where t' is the relative retention time observed with a selective stationary phase and t'_n is the relative retention time observed with a non-selective stationary phase. Woodford and van Gent had employed polar and non-polar columns to study the behavior of saturated fatty acids.

T values of androstane isomers (Table 17) calculated from measurements obtained on the XE-60 and the SE-30 phases indicate the dominating influence of the C-17 oxygen function in the 5β-isomers when the C-3 configuration is of the alpha type. Thus T values might be useful in reflecting stereochemical relationships of functional groups and might provide clues for intramolecular interactions.

The ketone selective properties of the four phases were compared and changes in retention time accompanying the oxidation of a hydroxyl group to the corresponding ketone were calculated (Tables 18 and 19). It is seen that the value of Δ log r is fairly constant and very small for SE-30, NGSeb and Hi-Eff 8B columns. Furthermore, there was no difference in the Δ log r value for ol $\rightarrow$ one and diol $\rightarrow$ dione transitions. The preferential selectivity of the XE-60 phase for ketones is evident in the relatively large values of log r_{ol_one} and in the greater retention time value on this phase of the 3-ketosteroids as compared to the 3-hydroxyl isomers (Table 18). The unexpectedly small values of Δ log r_{diol_dione} may be a direct consequence of the anomalous effect of C-17 substitution. However, it is also possible that some loss of the XE-60 phase may be the cause of the unexpectedly small value of these diol-dione transformations.

In Table 20, where separation factors for the 5α- and 5β-isomers are given, it can be seen that these separations are in general better for the trimethylsilyl derivatives, especially within the 17-ketosteroid group.

From the comparative data presented it is evident that the gas-liquid chromatographic separation of almost any isomeric pair can be achieved by the judicious choice of the stationary phase or of the derivative of the steroids under study. It is worthy of note that the isomeric 3,17-dione androstanes which have eluded separation by paper chromatography methods, can be resolved on gas-liquid chromatography columns coated with polar liquids (Table 11).

The Application of Gas-liquid Chromatography to the Isolation and Measurement of Urinary 17-Ketosteroids

A Method for the Simultaneous Separation of $C_{19}O_2$ and $C_{19}O_3$ 17-Ketosteroids and Progesterone Metabolites (100)

The term "urinary 17-ketosteroid" has been used for a group of substituted androstanes excreted by man. During the last twenty years a large body of data has become available on the excretion of 17-ketosteroids. Bioassay methods, although necessary, have been limited in usefulness because the steroids are excreted in a less active form or in the inactive form of their hormonal precursers. The chemical method used most widely for the determination of 17-ketosteroids is the assay based on the Zimmermann reaction (57,58). Since the urinary 17-ketosteroids are end products of the metabolism of hormones secreted principally by the adrenal cortices (115-118) and the gonads (119,120) any method which can measure the amounts of the 17-ketosteroids may also indirectly help to assess the gonadal and adrenocortical functions in man.

The excretion of 17-ketosteroids in urine is influenced by the basal metabolic status of the individual as well as by conditions of stress. The former includes age and sex of the individual while

the latter term may involve chronic stress resulting from a disease or a shorter term emotional or physical stimulus. The 17-ketosteroid excretion at any one time reflects the combined contributions of all of these factors.

The array of excreted C_{19} steroids, the urinary 17-ketosteroids, was first disclosed by the chromatographic studies of Dingemanse et al. (121,122). Subsequently moderately successful separations of 17-ketosteroids by adsorption and partition systems were accomplished (65,123, 124). While many conventional chromatographic techniques for the separation and measurement of individual 17-ketosteroids have been developed and are in actual use today, all suffer from the disadvantages of tediousness and inefficiency. The classical column chromatographic method (121,122) required the collection of multiple discreet samples of eluent each of which then had to be analyzed colorimetrically using the Zimmermann reaction (57,58). This reaction is at best a non-specific chromogenic reaction subject to interference by non-steroidal impurities. The paper partition method of Savard (65) has represented an improvement in efficiency in that the actual number of samples to be finally eluted and measured as Zimmermann chromogen are limited to those being resolved and studied. However, running times are long in these systems. By virtue of very short running times, thin layer partition chromatography will obviate this disadvantage. All of these modalities have a common limitation in resolving power attributable to practical limits to which the chromatographic column,

plate or paper strip can be lengthened. Specifically one encounters difficulty in trying to separate more than five or six components at a time by any of these liquid-liquid methods.

Following the demonstration of the feasibility of gas-liquid chromatographic analysis of steroids (83,86,112) concerted efforts have been directed to the development of methods for the analysis of the urinary C_{19} steroids. The first attempts involved the simultaneous use of two columns (125,126). The disadvantages were found to be those inaccuracies and inconveniences inherent in such a complex system. Partial separation of urinary 17-ketosteroids on SE-30 column have been reported (127). More recently separation of three 17-ketosteroids was reported on a cyanomethylsilicone column (107). However, a comprehensive method of analysis should separate and measure at least seven urinary end products, which are: 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one, 3β-hydroxyandrost-5-en-17-one, 3α-11β-dihydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-11,17-dione, 5β-pregnane-3α,20α-diol and 5β-pregnan-3α-ol, 20-one.

A review of the results obtained in the first phase of the study indicated that the trimethylsilyl ethers were the most efficacious derivatives for biological application. This was attributed to their high volatility and fast elution time with subsequent sharp symmetrical peaks. Separation factors for the trimethylsilyl ethers of the

steroids were also more favorable than for the free steroids or for the other derivatives tested. In addition, column efficiencies were generally higher for these derivatives. When mixtures of trimethylsilyl ethers of the C_{19} steroids and of two progesterone metabolites were chromatographed on the column phases described earlier, adequate separations were obtained in the case of the three per cent XE-60 and the one per cent Hi-Eff 8B columns; NGSeb column provided only marginal resolution of the 3α-hydroxy-5β-androstan-17-one and 5β-pregnane-3α,20α-diol. The results are shown in Table 21 and are graphically depicted in Figures 9-11. Thus the method developed analyzed not only the five major C_{19} steroids but in addition two progesterone metabolites of great interest in human metabolism.

In an actual application of this basic technique to the study of human urine it was found that the initial objectives of the investigation could be realized. Chromatography on a three per cent XE-60 column of the trimethylsilyl derivatives of a glusulase hydrolyzed and solvent extracted 24 hour urine sample from a normal male subject gave the results shown in Figure 12. By comparison of retention times for major peaks with those obtained for pure crysalline standards it was possible to quantitatively detect and identify the urinary components. Although no attempt at measurement was made, the distribution and the relative proportion to each other of these compounds, represented as peak height responses should be noted. Since the chromatogram was obtained using a 1/2040 aliquot of the urine sample, the indications are that the method will permit the requisite

sensitivity for determinations at both the physiological and pathophysiological levels. Indeed it is conceivable that plasma levels of these steroids may be determined by this technique.

Advantages of the present method include the factor of speed, efficiency and a one-step process of separation and measurement. The complete analysis of a prepared extract can be accomplished in 25 minutes as compared to the seventy-two hours necessary for the development of a paper chromatogram.

The success of the biological application supported the original premise that access to a useful analytical technique may come from a thorough knowledge of the compounds to be studied and from the generalizations gained in the investigation of their properties which in the present research was their chromatographic behavior.

Summary

The relative polarities of the four stationary phases employed were established as Hi-Eff 8B > NGSeb > XE-60 > SE-30. The general pattern of retention times of the free steroids and their derivatives were: acetate > trimethylsilyl ether > free steroid > trifluoroacetate on the SE-30 column; acetate > free steroid > trimethylsilyl ether on the XE-60 column; free steroid > acetate > trimethylsilyl ether > trifluoroacetate on the Hi-Eff 8B and NGSeb columns.

On all four columns 5α-androstanes showed a greater retention time than the corresponding 5β-isomers. This was attributed to the planarity of the 5α-androstane nucleus and enhanced interaction with the stationary phase. In agreement with earlier observations on paper partition systems, the mobility of the axial isomer was greater than its equatorial isomer. In the 5β-androstane series, the steroids with an oxygen function at C-17 exhibited anomalous behavior in that they had greater retention times than the corresponding 5α-isomers. The absence of the anomalous behavior in the 5β-androstanes containing oxygen substituents in both the C-3 and C-17 positions indicated the opposing influence of the C-3 oxygen function.

Among the three derivatives the acetates were found to be the most satisfactory in correlation of chromatographic behavior to structure. The best resolutions were affected with the trimethylsilyl ethers of the steroids.

Separation of the principal urinary 17-ketosteroids was achieved on one per cent NGSeb, one per cent Hi-Eff 8B and three per cent XE-60 columns. The two pregnane isomers studied were resolved simultaneously with the 17-ketosteroids on the latter two columns.

The gas-liquid chromatographic method developed was successfully applied to the separation of the major 17-ketosteroids on a crude urine extract.

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BIOGRAPHICAL SKETCH

Iclal Sirel Hartman was born December 22, 1930 at Elazig, Turkey. She attended Preparatory School in Istanbul, Turkey and received the degree of Bachelor of Arts in June 1950 from Mount Holyoke College. In June, 1951 she received the degree of Master of Arts from Mount Holyoke College. From 1952 to 1955 she was employed by E. R. Squibb and Sons, first as a trainee and later as the Head of the Quality Control Department in Squibb Istanbul. In 1955, she enrolled in the Graduate School of the University of Florida. She interrupted her graduate study to teach. She joined the faculty of Simmons College in 1959 where she is at present teaching Chemistry. She resumed her graduate study in 1961 and since that date has pursued her graduate work toward the degree of Doctor of Philosophy simultaneously with her teaching.

Iclal Sirel Hartman is married to Standish Chard Hartman, Jr.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 20, 1963

Dean, College of Arts and Sciences

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Supervisory Committee:

Chairman

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